

Department of Chemistry
Universität Freiburg (Schweiz)

Chlorophyll Catabolism in Algae and Higher Plants

A Chemical Approach

Habilitationsschrift zur Erlangung der *venia legendi*
an der Mathematisch-Naturwissenschaftlichen Fakultät der
Universität Freiburg in der Schweiz

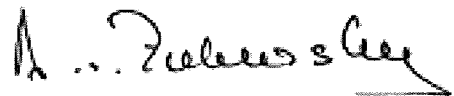
von
Dr. Norbert G. Engel
aus Berlin

Freiburg (Schweiz) 2001

Von der Mathematisch - Naturwissenschaftlichen Fakultät angenommen

Freiburg, den 10.01.2002

Der Dekan

A handwritten signature in black ink, appearing to read 'A. von Zelewsky', with a horizontal line drawn under the last part of the signature.

Prof. Dr. Alexander von Zelewsky

Department of Chemistry
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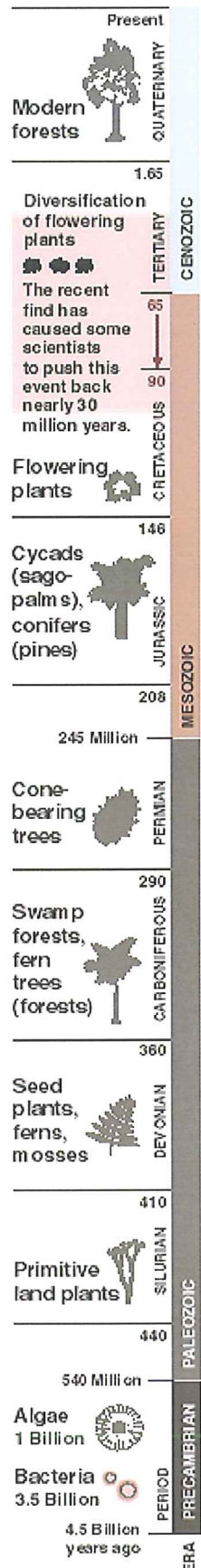
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CONTENTS

ZUSAMMENFASSUNG	1
SUMMARY	2
INTRODUCTION	
Chlorophylls and Hemes: the "Pigments of Life"	3
HISTORICAL SURVEY	
Chlorophyll Degradation in Green Plants	5
Structures of the Chlorophylls	8
Biosynthesis of Chlorophylls and Hemes	9
Biological Considerations	10
Predictions of the Structures of the Catabolites	13
STATE OF THE RESEARCH	
Chlorophyll Catabolites from Different Organisms	15
Marine organisms	15
Freshwater green algae	16
Higher plants	19
Enzymatic and Chemical Reaction Mechanisms	20
Primary ring cleaving step	21
Terminal step of the primary ring cleavage	22
Pyrroline to pyrrole-ring transformation	23
Catalytic hydrogenation	25
Enzymatic <i>ex vivo</i> systems	27
Chlorophyll <i>b</i> to chlorophyll <i>a</i> reductase	28
Green Algae as Progenitors of the Higher Plant Cell	30
Tentative Degradation Pathway	31
FUTURE PROSPECTS	35
ACKNOWLEDGEMENTS	36
APPENDIX	37
REFERENCES	39
PERSONAL PUBLICATIONS	45
ACCEPTED DISSERTATIONS	47
PHOTOCOPIES OF ORIGINAL PUBLICATIONS	70 PAGES

This postdoctoral thesis focuses on the chemical aspects of endogenous chlorophyll degradation in algae and higher plants and concentrates on the results accomplished in the Department of Organic Chemistry of the University of Fribourg, Switzerland. Biological, enzymatic, and genetic aspects of chlorophyll degradation are only referred to, when necessary, to decipher chemical aspects. Additional information, especially experimental details, spectroscopic data and an exhaustive literature survey can be extracted from the original publications attached to this document.

ZUSAMMENFASSUNG

Jährlich werden schätzungsweise 1000 Millionen Tonnen Chlorophyll im Pflanzenreich abgebaut. Das scheinbar spurlose Verschwinden dieser auffälligen und für das Leben so wichtigen photosynthetischen Pigmente war bis vor kurzem unerklärlich. Erst 1991 wurden die ersten Chlorophyllkatabolite aus Pflanzen und Grünalgen isoliert und deren Strukturen durch spektroskopische Methoden ermittelt.

Aus dem Kulturmedium der Grünalge *Chlorella protothecoides* konnten durch den Wechsel von photoautotrophen zu heterotrophen Wachstumsbedingungen rote Pigmente isoliert werden, deren Strukturen eindeutig ihre Herkunft aus den Chlorophyllen *a* und *b* belegen. Aus grünen abgetrennten Primärblättern der monokotyledonen Pflanze *Hordeum vulgare* (Gerste) wurden farblose Chlorophyllkatabolite isoliert, wobei der Chlorophyllabbau durch permanente Dunkelheit ausgelöst wurde. Inzwischen sind weitere Katabolite aus höheren Pflanzen isoliert worden, einschliesslich aus den herbstlichen vergilbten Blättern von Bäumen (*Liquidambar spec.* und *Cercidiphyllum japonicum.*). Alle bisher aus grünen Pflanzen isolierten und charakterisierten Chlorophyllkatabolite sind lineare Tetrapyrrole, die durch einen regioselektiven, oxygenolytischen Bindungsbruch an der Position C₄=C₅ der ursprünglichen Chlorophylle entstehen. Mit Hilfe von *in vivo* Markierungsexperimenten mit *C. protothecoides* konnte durch Sauerstoffisotope die Beteiligung einer Monooxygenase im ersten Schritt der Ringöffnungsreaktion nachgewiesen werden. Durch Verwendung von schwerem Wasser wurde in *Chlorella* der hohe stereoselektive Ablauf im terminalen Schritt der Ringöffnung, und in höheren Pflanzen die dem Abbau vorausgehende Umwandlung von Chlorophyll *b* in Chlorophyll *a* belegt werden. Das rote Hauptpigment aus *C. protothecoides* konnte chemisch durch katalytische Hydrierung und nachfolgende protonenkatalysierte Umlagerung in das Grundgerüst der farblosen Pflanzenkatabolite transformiert werden. Die bisherigen Ergebnisse deuten auf einen allgemein gültigen Abbauweg der Chlorophylle in der Entwicklungslinie der grünen Pflanzen hin.

SUMMARY

Every year about 1000 million tons of chlorophylls are degraded in the plant realm on earth. The disappearance of these salient photosynthetic pigments, which are indispensable for life, was for long regarded as an enigma. As late as 1991 the first chlorophyll catabolites from plants and green algae were isolated and structurally elucidated by spectroscopic means.

Red pigments whose structures originate from chlorophyll *a* and *b* were isolated from the culture medium of the green alga *Chlorella protothecoides* when switched from photoautotrophic to heterotrophic growth. From green freshly cut primary leaves of the monocot barley (*Hordeum vulgare*), colorless chlorophyll catabolites were isolated. The degradation was induced by permanent darkness. Meanwhile, several new catabolites from higher plants have been characterized including autumnal yellowing leaves of the deciduous trees (*Liquidambar spec.*, *Cercidiphyllum japonicum*). All catabolites isolated so far are linear tetrapyrroles which arise from a regioselective oxygenolytic ring-cleavage at position C₄=C₅ of the original chlorophyll. *In vivo* labeling experiments of *C. protothecoides* with oxygen isotopes evinced the involvement of a monooxygenase in the primary step of the chlorophyll catabolism. *In vivo* labeling experiments of *Chlorella* and barley with heavy water demonstrated the high stereoselectivity in the terminal step of the primary ring opening mechanism and showed that in higher plants Chlorophyll *b* is converted to chlorophyll *a* before degradation. Catalytic hydrogenation and subsequent prototropic rearrangement transformed the red pigments isolated from *C. protothecoides* into the basic skeleton of the colorless plant catabolites. The results obtained so far suggest an existing general catabolic pathway of the chlorophylls in green plant lineage.

INTRODUCTION

Chlorophylls and Hemes: the "Pigments of Life"

Chlorophylls (Chls) and hemes represent a group of tetrapyrrolic pigments with cognate structural elements with antagonistic functions. Chls are magnesium complexes of cyclic tetrapyrroles of the porphyrin, chlorin or bacteriochlorin oxidation state. An isocyclic ring system is generally fused to the tetrapyrrolic moiety, which originates from one of the former propionic acid side chains of protoporphyrin IX. Most of the chlorophylls are esterified with a lipophilic terpen-alcohol, commonly phytol. The function of the chlorophylls is to capture solar light energy and to convert the energy of the absorbed photons into excited electrons, eventually into a stabilized chemical potential. The major pigment is Chl *a*, which is ubiquitous in the reaction centers of all oxygen-evolving photosynthetic organisms in which water is the ultimate electron source (Fig. 1).

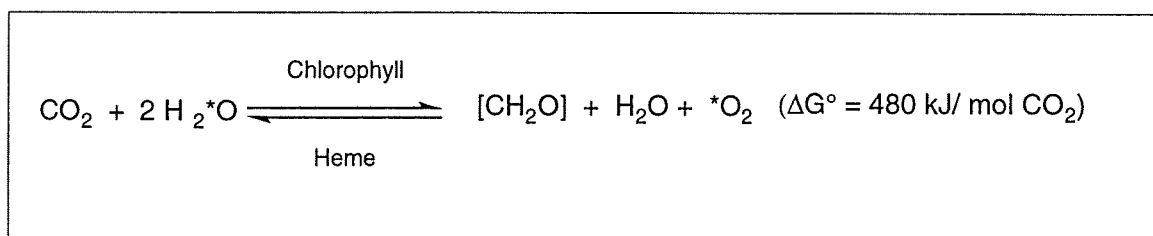


Fig. 1 The basic reaction scheme shows the involvement of the chlorophylls and hemes in the process of energy transduction. The energy for the synthesis of carbohydrates, the primary storage material of plants and oxygen are supplied by solar radiation. Both components are utilized to sustain heterotrophic life of plants and animals. Asterisks indicate the fate of oxygen.

In green algae and higher plants the light harvesting complexes contain chlorophyll *b* and carotenoids as accessory pigments in addition to chlorophyll *a*. The chlorophyll-protein complexes are orderly assembled in the antennae systems and reaction centers of the photo-organisms. The

photosynthetic apparatuses are located in the thylakoid membranes of the chloroplast(s).

Hemes on the other hand are iron complexes of porphyrins. In cytochromes, these pigments function as electron carriers, which pass electrons - originating from the light reaction of chlorophylls - back to oxygen. The cytochromes are assembled as protein complexes in the inner membrane of the mitochondrion. Both chlorophylls and hemes are fundamental for the continuation of the life cycle. The latter releases the energy captured by the former. Owing to their great impact on the sustenance of life, they have been termed by A. Battersby as - Pigments of Life - [1].

HISTORICAL SURVEY

Chlorophyll Degradation in Green Plants

While chemical structures, biosynthesis and function of the chlorophylls are known in detail, chlorophyll catabolism, on the contrary, has remained one of the least well understood biological processes and was until recently only a matter of speculation. The phenomenon is particularly obvious during autumnal leaf coloring of deciduous trees and shrubs in which the leaves lose their greenness and turn yellow, gold and red. These color changes can even be observed from outer space [2]. Heme catabolism on the other hand has received considerable attention for the obvious reasons of human health care, beginning with the research of H. Fischer [3].

It has been estimated that annually about 10 million tons of heme related compounds are degraded by heterotrophic organisms into bile pigments, whereas much more, about 1000 million tons of Chls are degraded in the plant realm [4]. Although the largest mass of chlorophyll is present on land, the greater part (75%) of the annual breakdown takes place in aquatic environments where the life span of many photosynthetic organisms is shorter. The disappearance of so much chlorophyll without the formation of detectable intermediates or end-products was for a long time regarded as an enigma. "The chlorophylls apparently disappear without leaving a trace" [5].

In the past, biologists such as J. Sachs (1863) [6], E. Stahl (1909) [7] and H. Molisch (1918) [8], to name a few, described the phenomenology and the environmental factors which influence the autumnal yellowing of leaves. E. Stahl noticed that the yellow leaves contained considerable lower amounts of "nitrogen" and other essential elements when compared to green leaves. Contemporary reviews by K. Simpson (1976) [9], R. Kufner (1980) [10],

G. Hendry (1987) [5], W. Rüdiger et al. (1989) [11] and recently by S. Brown et al. (1991) [12] brought the wide spread knowledge into focus. All authors stress the necessity of understanding this phenomenon from both the fundamental as well as the applied viewpoint.

Metabolic pathways in living organisms are generally divided into two categories: those involved in the biosynthesis (anabolism) and those involved in the degradation (catabolism) of bio-molecules. In the common catabolic pathways complex molecules such as carbohydrates, lipids and proteins are stepwise degraded into diverse substances, which are re-utilized as starting material for the synthesis of essential cell components or for energy production. The term catabolism, degradation and decomposition are likewise used to indicate enzymatic degradation of exogenous and endogenous compounds, for example during morphological differentiation, environmental adaptation and/or detoxification. Commonly, superfluous molecules are enzymatically degraded. Generally this happens by oxidative processes through the action of non-specific enzymes containing heme as a prosthetic group, as for example the enzymes of the cytochromes P-450 family. Specific enzymes such as heme oxygenase degrade heme to the well-known bile pigments. The terminal products of degradation are either excreted or stored directly, occasionally after derivatization, in the vacuoles of the (plant) cell.

In contrast to the peripheral substituents, the chlorophyll macrocycle with its highly extended electronic system is remarkably stable, particularly when oxygen and light are excluded. Some metal complexes of phorbins have survived chemical modifications over a long geochemical time scale and thus survived as "molecular fossils" in sediments dating back to the Precambrian period [13]. Chlorophyll breakdown has been induced in intact plant tissues by chemicals, plant hormones [14] and by permanent darkness, for example in *Euglena* (*Euglena gracilis*), [15] barley *Hordeum vulgare*, [16] in cell cultures of

tobacco (*Nicotina tabacum*) [17] and in parsley (*Petroselinum crispum*) [18]. In addition, exogenically applied randomly labeled chlorophylls were injected in bell peppers (*Capsicum frutescence*) [19]. In all these experiments only peripherally altered degradation products as for example pyropheophorbide *a* were detected; macrocyclic ring cleavage products as suggested by Hendry have never been found in green plants before 1991.

Cells may die by apoptosis (programmed cell death) or by accident. Chlorophylls are not only degraded during autumnal foliar senescence but also in a variety of other situations, for example during fruit ripening, chlorophyll turnover during the vegetation period and by activities of herbivores. Exogenic chlorophyll catabolism in animals has been investigated since the times of H. Fischer in which peripheral modified porphyrinoides such as phytoporphylin were characterized in faeces from elephants, cattle, goats and horses and from the gall bladder of oxen [3,20]. Within humans only rudimentary studies were performed [21].

Structures of the Chlorophylls

In 1883, Berzelius extracted the green pigments from the leaves of plants [22]. Stokes discovered that the extracts consisted of two components "green substances exhibiting strong red fluorescence" [23]. Twett separated both pigments for the first time using chromatographic techniques. Willstätter determined the correct molecular formulas of Chl *a* and *b* [24] and observed the action of an enzyme which was named chlorophyllase. This enzyme cleaves the phytol ester group of chlorophyll *a* [25]. H. Fischer elucidated the constitutions of most natural Chls as well as the related porphyrins. The constitution of the Chl *a* was confirmed by the Woodward group through total synthesis of the base Chl *a* skeleton using rational reaction sequences [26]. Fleming [27] and Brockmann [28] finally determined the absolute configuration of the three stereogenic centers of the Chl macrocycle.

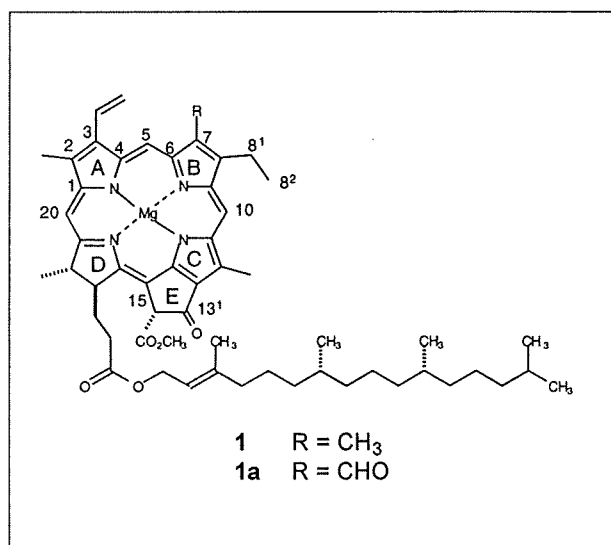


Fig. 2 Structure of the most abundant natural chlorophylls *a* R=CH₃ and *b* R=CHO showing the absolute configuration of the three stereogenic centers on the macrocycle C₁₃² (R), C₁₇ (S), C₁₈ (S) and the phytol side chain[†]

[†] Since the systematic names used in Chemical Abstract for chlorophyll degradation products are too cumbersome for practical purposes, semi-systematic names based on the basic ring system were used throughout this work. Examples of the nomenclature of the chlorophylls and related compounds are shown in the appendix.

The large ring system is composed of three pyrrole units and a pyrroline ring moiety (Fig. 2). Together they constitute an extensive system of 18π -conjugated double bonds, which is comparable to the aromaticity of a [18]-annulene system. Characteristic for most Chls is a fifth isocyclic ring fused to the Mg-containing macrocycle. The proton connected to C₁₃² is part of a β -keto-ester structure and therefore is prone to epimerization and allomerization reactions during the isolation procedures.

Biosynthesis of Chlorophylls and Hemes

A major advance in the elucidation of the biosynthetic pathways of the porphyrins was achieved with the advent of commercially available isotopes. The pioneering research of Shemin and co-workers demonstrated that in animals all hemes are synthesized from two components, glycine and succinylCoA [29]. The methods used were later extended to the study of the biosynthetic pathways of the Chls.

It has been amply demonstrated that in higher plants, green algae, red algae and cyanobacteria all cellular tetrapyrroles (Chls and hemes) are biosynthesized from the intact carbon skeleton of glutamate by the so called - five carbon pathway - [30]. The key-intermediate of all porphyrins in all organisms is 5-aminolevulinate.

Two molecules of 5-aminolevulinate are condensed by ALA-dehydrase to form porphobilinogen (PBG). Four molecules of PBG cyclize to form uroporphyrinogen III in a complex reaction sequence in which a dipyrromethane cofactor is involved. Subsequent side chain modifications and eventual oxidation of the porphyrinogens yields protoporphyrin IX. Chelation of this branch-point macrocycle with either magnesium or iron

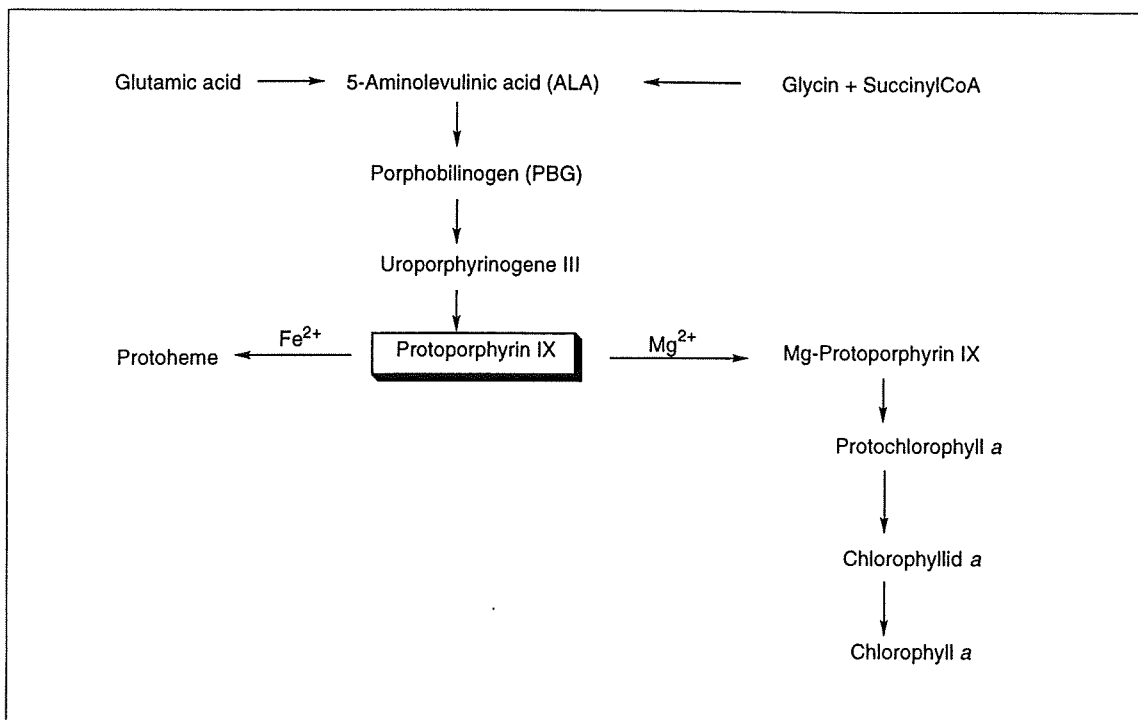


Fig. 3 The biosynthetic pathway of tetrapyrroles. This outline is focused on the starting materials and the branch point leading to hemes or Chls, respectively.

determines the further progress of the biosynthesis, leading to the Chls and hemes, respectively [31].

Biological Considerations

In leaf senescence the environmental stimuli are the shortening days and cooler temperatures of autumn. It is important for perennial plants that live for many years to anticipate unfavorable conditions before they actually arrive. The final loss of leaves each autumn is an adaptation that keeps deciduous trees from desiccating during winter when the roots cannot absorb water from the frozen ground. In the autumn leaves new Chl synthesis is arrested. The color change from green to yellow is due to the preferential degradation of the Chls; the colors of the yellow carotenoids commonly present in leaves become visible. *De novo* synthesis of new compounds such as anthocyanins and phenolics are responsible for the red and brown colors of autumnal leaves.

During autumnal climax, particularly nitrogen containing compounds but also other rare elements as Fe, P, and K are relocated into storage tissue in a precisely timed active process [7,32]. Storage tissues are the seeds in monocarpic plants, or buds and roots in polycarpic plants. Newer quantitative investigations have shown that during senescence of annual and perennial plants cellular "nitrogen" is released from existing proteins by proteolysis and transamination reactions of the constituting amino acids. Delocalization proceeds from the leaves to the storage tissue of the plant (Fig 4).

Foliar senescence starts with a programmed disintegration of the chloroplasts. Particularly the functional chloroplast is considered an important storage organelle. 70-80% of the nitrogen in mature leaves are located in the chloroplast [33]. Roughly, 90% of the nitrogen exported from senescing leaves are from the chloroplast [34]. The apo-proteins of pigments in photosystems and light harvesting complexes represent 30% of the total chloroplast nitrogen while RuBisCo comprises up to 50%. The amount of nitrogen bound in the Chls can be neglected; it amounts to only 4% of the whole-reduced nitrogenous components in plant cells [35].

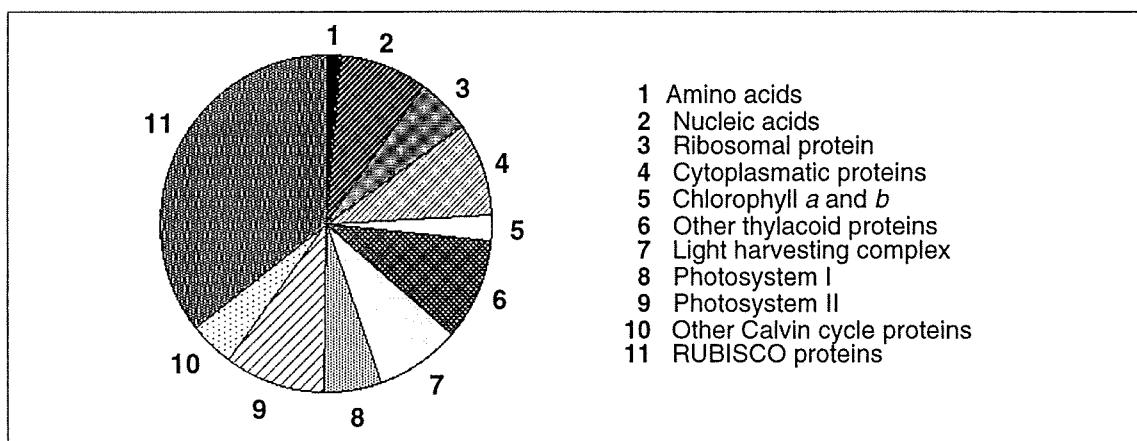


Fig. 4 Distribution of reduced nitrogen among various proteins and other nitrogenous compounds in a mesophyll cell. The drawing is adopted from Peoples M. and Dalling M [36].

Chls or its degradation products which might arise during the degradation of Chl-protein complexes (*vide infra*) are considered to be photodynamically active. Degradation of the proteins of the photosynthetic units to amino acids without destroying these chromophors could possibly liberate dangerous photodynamic molecules. Therefore, it has been suggested that the degradation of the Chls serve to avoid the accumulation of toxic photodynamic pigments during the nitrogen-relocation process. For plants it is essential to eliminate or to convert such products rapidly into colorless compounds, otherwise the cells become damaged under strong light. Castelfranco and Beale have discussed a similar situation for Chl precursors [37,38]. Therefore, it is not surprising that in higher plants the primary catabolites are reduced in such a way, that a chromophoric system is no longer present and photodynamic destruction of the cells is impossible.

A further modification such as esterification with malonate or ligation to glycosides seems to increase the water solubility of the catabolites and appears necessary for intercellular trafficking. Plant catabolites are eventually disposed in the plant vacuole [32]. Some plants, including gymnosperms and most algae, do not require light for Chl formation; such organisms are not expected to degrade Chls in darkness.

Degradation of the Chls appears to be of evolutionary advantage for those plants, giving them the necessary time to relocate and store nutrients that are used in spring for germinating, flowering and greening-up. The ultimate fate of the Chl catabolites after leaf fall is unknown but certainly a matter of bacterial biodegradation. Leaf senescence is now recognized as a highly controlled sequence of biochemical and physiological events comprising the final stage of development, from mature fully expanded state until death. During apoptosis, the cells remain viable and new gene expression is required.

C. Smart presents the current understanding of senescence at the organelle and molecular levels in a review [14].

In algae a similar process might occur. Glucose bleaching of *C. protothecoides* is induced under nitrogen starvation and proceeds in darkness as well as in light. Under conditions of nitrogen starvation, the cells start protein degradation to provide necessary nitrogen for indispensable metabolic processes. Proteins of the chloroplasts, no longer necessary under heterotrophic conditions, are degraded. This auto-digestive process would supply the essential nitrogen for cell maintenance and propagation. The degradation of the Chls in *C. protothecoides* is extremely fast and is completed in about 56 hrs (*vide infra*). The lipophilic Chls are converted by oxygenation into hydrophilic (red) catabolites, making them water-soluble and therefore apt to excrete. It is characteristic of algae, where diffusion of metabolites into the surrounding is facilitated, that they have less developed oxygenase systems than higher plants. As has been determined, the red algal pigments are still photodynamic active; they sensitize the photo-oxygenation of biphenyl anthracene in organic solvents under self-destruction [39].

Predictions of the Structures of the Catabolites

Calculation of the electronic structure of chlorins in the Hückel approximation of the method of molecular orbitals show that only position C15 and C20, adjacent to the pyrroline ring moiety, carry an excess of π electrons [40]. The calculations of Wheland's localization energies entirely confirm these results. When the position C15 is blocked as in Chl, the C20 will remain the only position reactive to electrophilic attack. These results are in accordance with the previous findings of the Woodward group, in which chlorins display a selective high reactivity toward electrophilic attack on C15 and C20 positions.

Positions C₅ and C₁₀ do not react under the same conditions[40,41]. On this basis, the C₂₀ methin-bridge adjacent to the reduced pyrrole ring D is preferentially attacked by electrophiles such as activated oxygen.

Bile-pigment-like linear tetrapyrroles have long been considered as Chl degradation products, in analogy to the well-known animal heme catabolism and plant phycobiline biosynthesis. In these processes, macrocyclic ring opening occurs by a microsomal heme oxygenase at position C₅ of protoheme [42]. This enzyme converts heme in a complex reaction sequence into the bile pigment biliverdin IX α in which the carbon atom C₅ is lost as carbon monoxide. Subsequent enzymatic reduction yields bilirubin IX α which is either excreted or further reduced in the intestine by micro-organisms to colorless bile-pigments. The different colors in the metabolites arise from different numbers of double bonds in conjugation. Consequently, it has been reasonably suggested that a similar ring opening mechanism followed by reductive processes may lead to colorless Chl degradation products, which are ultimately degraded into nitrogen and carbon dioxide [5]. Curiously, a transformation sequence of the carbon skeleton of the Chls into secondary carotenoids was still suggested as late as 1968 [43].

STATE OF THE RESEARCH

Chlorophyll Catabolites from Different Organisms

Chl catabolites have been isolated from marine organisms, fresh-water green algae and from angiosperms. Two types of Chl degradation products have been hitherto isolated: products in which macrocyclic ring opening occurs at the C₁=C₂₀ bond and such in which the position C₄=C₅ is oxygenolytically cleaved.

Marine organisms

The first natural bile pigment (2) (Fig. 5) structurally related to Chl was isolated from euphausiid krill *Euphausia pacifica* in 1988 [44]. The shrimp-like animals lack photosynthetic capabilities, the compound must therefore arise from the Chl content of the algae prey.

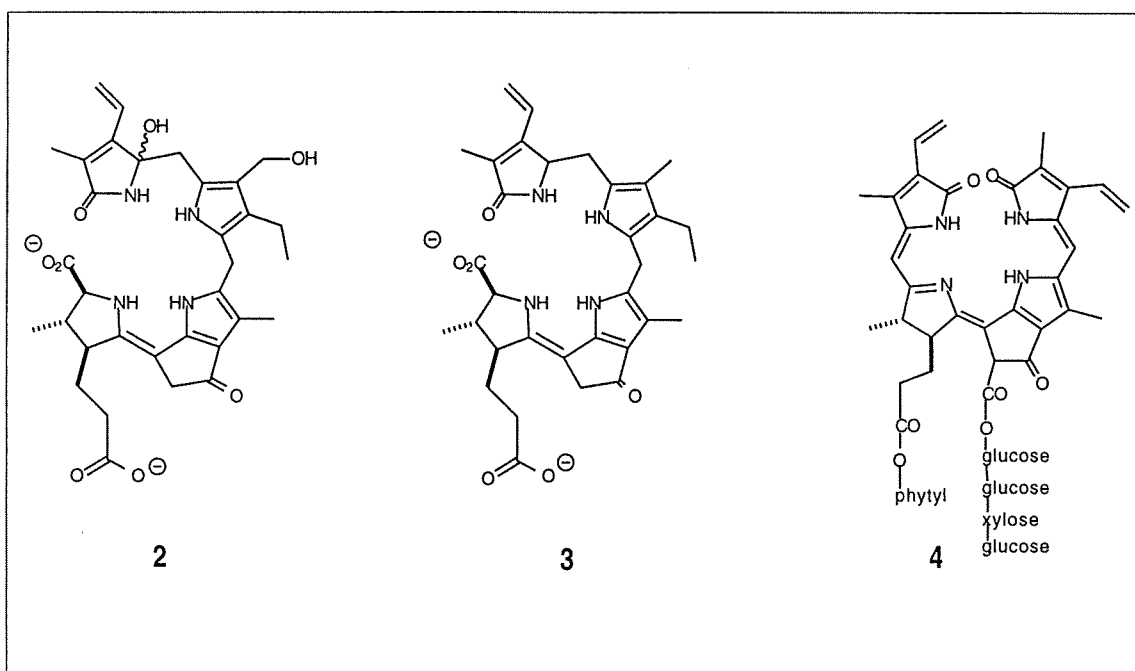


Fig. 5 Chl-degradation products isolated from marine organisms. 2 Substance F from *Euphausia pacifica* [44]; 3 Dinoflagellate luciferin from *Pyrocystis lunula* [45] and 4 from *Bryopsis maxima* [46].

A similar tetrapyrrolic compound (3) was found in the dinoflagellate *Pyrocystis lunula* [45]. Although the latter organism is capable of photosynthesis it contains Chl *d* as accessory pigment instead of Chl *b*. Taxonomically, members of this division are not considered as green plants. Notably, fluorescence is observed in both compounds, which enables the organisms to bioluminescence. In contrast to the catabolites isolated from green plants, both compounds are oxygenolytically cleaved at positions C₁=C₂₀. The former methine-carbon-atom C₂₀ of the Chl is retained as C₂₀-carboxylate on ring D, conjugation of the pyrrole moieties is interrupted; both compounds are, as expected, colorless.

A tentative structure 4 was proposed for the main red pigment of the catabolite isolated from the green alga *Bryopsis maxima* [46]. This compound has retained the phytol ester group present in Chl and has a sugar chain comprised of xylose and glucose attached to the carboxylate group at C₁₃²; no aldehyde group has been found at C₅. However, UV/vis spectroscopic data presented are not consistent with a fully conjugated linear tetrapyrrole and several other structural assignments are rather ambiguous.

Freshwater green algae

Chlorella algae are generally microscopically small spherical cells (3-10 µm in diameter) which propagate asexually through autosporulation. When grown under autotrophic conditions they contain one cup-shaped chloroplast. They are ubiquitous and found mostly in sweet water, they occur also as symbionts in animals and as algae components of lichen. In the surveys of the metabolic processes in higher plants, algae were often studied first. One reason for this is the relative simplicity of cell organization, as compared with higher plants. Second, algae illustrate with great clarity many important phenomena that in other plants are complicated. The third reason is simplicity of manipulation

when compared with vascular plants. *Chlorella* algae are easily cultured under submerge conditions and grow independent of seasonal changes.

Chlorella protothecoides Krüger ACC 25 has been used extensively throughout this work. It is a unique photosynthetic organism which can easily adapt to heterotrophic as well as autotrophic modes of growth [47,48]. When an organic nutrient such as glucose or acetate is available in the growth medium, this alga shifts from autotrophic to heterotrophic mode of growth. Under the heterotrophic mode of growth not only any further development of chloroplasts is suppressed but also the already existing photosynthetic membranes undergo disintegration. The cells become entirely colorless (Chl-less) and excrete red pigments into the culture medium. Once the heterotrophic nutrient present in the medium is depleted, the bleached cells reversibly turn green in light on provision of a nitrogen source. Although these red pigments were at the time of its discovery regarded as Chl catabolites definite structures have not been established [48].

At Fribourg the structure of the methyl ester of the main red degradation product of *C. protothecoides* **7a** (Fig. 6) was finally elucidated by ^1H and ^{13}C -NMR-spectroscopic methods [49], X-ray crystal diffraction [50] and chemical partial-synthesis [51]. The compound exhibits fluorescence at 663 nm with an efficiency of $\Phi = 2.0$ [39].

Soon after the discovery of the main Chl degradation product, the $\text{C}_{15}=\text{C}_{16}$ -*E*-isomer **8** was identified together with derivatives of the Chl *b* series (**6**, **6a**) [52,53]. Isomerization has been chemically induced under mild alkaline condition in which the *E*-isomer is predominant and therefore considered as thermodynamically more stable. Later it was demonstrated that the original Chl catabolites carry a carboxylate group at position C_{13}^2 [54].

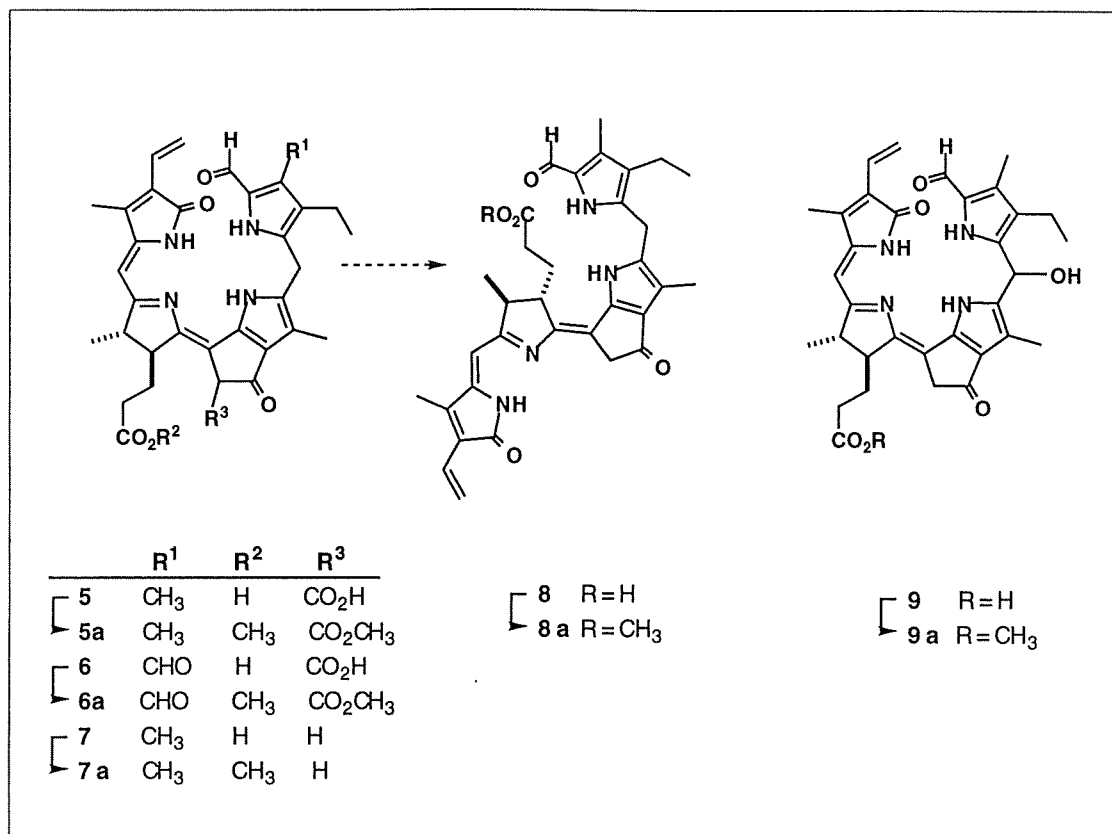
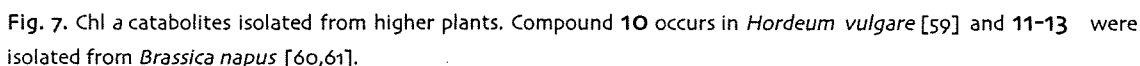


Fig. 6 Catabolites derived from Chl *a* 5 and *b* 6 were isolated from *Chlorella protothecoides* and were characterized as dimethyl ester derivatives 5a and 6a, respectively. Formulae 7 show the Z-isomer and its corresponding methyl ester 7a obtained by an acid work-up procedure of the culture medium. The analogous E-isomer 8 was acquired by alkaline isomerization of 7. Subsequent esterification with methanol afforded methyl ester 7a. Formula 9 shows a structurally related pigment which was isolated from *C. kessleri* and chemically transformed to 9a.

Later it was shown that the carboxylate group is easily lost in a non-enzymatic process during the work-up procedure (decarboxylation of the free β -keto acid). Up to 60% of the original Chls contained in the green cells were recovered as methyl ester derivatives 5a and 6a, both were found in a relation of about 3 : 1, respectively. This figure reflects the normal relation of Chl *a* to *b* found generally in green algae cells. From the green alga *Chlorella kessleri*, a similar Chl *a* degradation product was obtained which carries an additional hydroxyl group at position C10 [55]. Recently a new red pigment has been isolated from a Chl-less *Chlamydomonas reinhardtii* mutant. Analysis of this degradation product by chromic acid oxidation, together with spectroscopic properties and molecular mass determination, indicated that the pigment

All Chl catabolites identified from angiosperms are colorless and non-fluorescent compounds. The compounds appear in ^1H -NMR as single diastereomers and display, as far as reported, a well-structured circular dichroism (CD) spectrum, thereby, exhibiting several Cotton effects [57,58].

Chlorophyll degradation in green excised primary leaves of barley (*H. vulgare*) is induced by watering the excised leaves for seven days in permanent darkness. Catabolite **10** (Fig. 7) was isolated from the plant extract by high-pressure liquid chromatography (HPLC) [59], and represents the first catabolite isolated from a higher plant.



From senescent dicotyledons of rape (*Brassica napus*) three similar compounds (**11-13**) were isolated which vary only in the substitution pattern of the C8² - β -hydroxyl group which is either non-substituted, esterified with malonate or was glycosylated with β -L-glucopyranose [60,61].

Catabolite **14** (Fig. 8) was isolated and identified from yellowing autumn leaves of *Liquidambar straciflua*, *Liquidambar orientalis* [62] and from *Cercidiphyllum japonicum* [39,58]. The presence of the bile pigment-like catabolite of the latter was detected throughout the enrichment procedure known as "chromic acid degradation", a method that is well established in porphyrin chemistry [63]. This oxidation method transforms pyrrole derivatives and oligo-pyrroles into the corresponding maleimide fragments. These maleimides are easily visualized on TLC plates by the highly sensitive chlorine/benzidine procedure, in which trace amounts of maleimides (<0.5mg) can be detected.

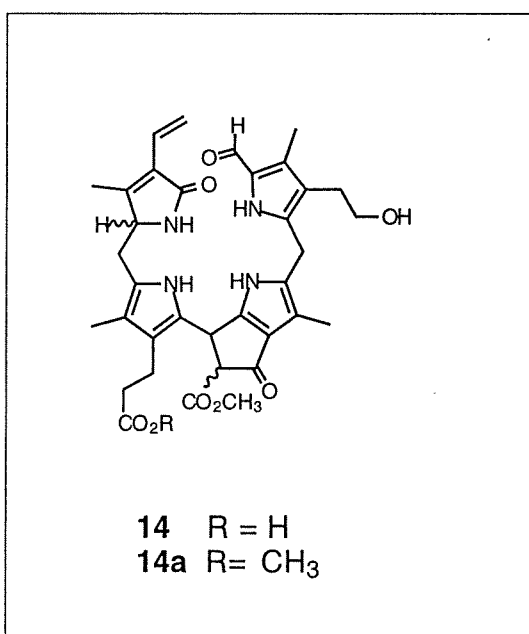


Fig. 8 Chl catabolite **14** was isolated from yellow leaves of deciduous trees of *Liquidambar spec.* [62] and *Cercidiphyllum japonicum* [58]. Compound **14a** is the product of a chemical esterification which facilitated the purification procedure.

Enzymatic and Chemical Reaction Mechanisms

In vivo labeling with oxygen isotopes and with heavy water was performed with *C. protothecoides* and several *ex vivo* experiments were implemented in order to gain inside into the cleaving mechanisms and the final process. Additionally, the acid catalyzed pyrrolin/pyrrole rearrangement of the main Chl-degradation product of *C. protothecoides* was studied in detail.

The Primary ring cleaving step

Chl catabolism in green algae and angiosperms occurs at the C₄=C₅ position, the position which is reminiscent of the heme catabolism. *In vivo* ¹⁸O-labeling experiments with mixtures of ^{18,18}O₂/^{16,16}O₂ in bleaching *C. protothecoides* and *C. kessleri* cells unequivocally demonstrated that in green algae the oxygen atom of the C₅-formyl group arises from molecular oxygen. The oxygen atom of the lactame group on the other hand originates from water (Fig 9) [64].

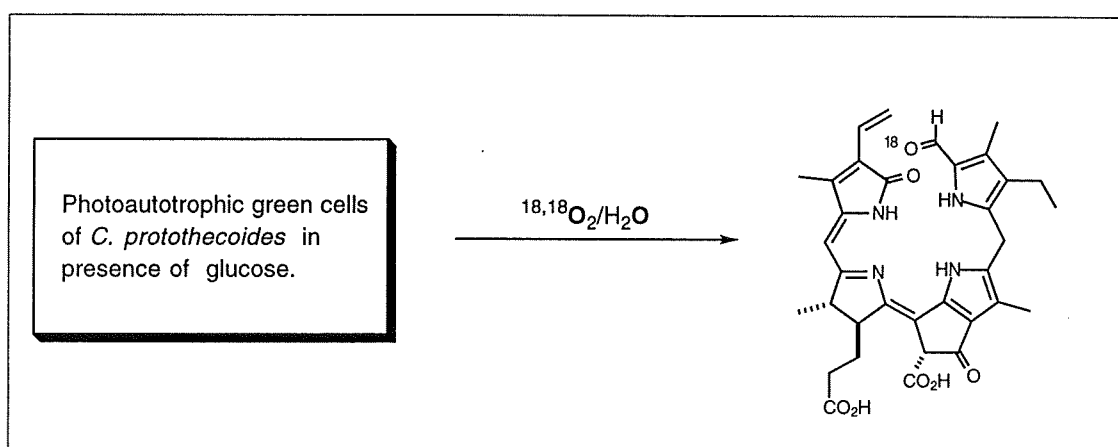


Fig. 9 Results of *in vivo* labeling experiments with *C. protothecoides* in the presence of isotopic di-oxygen mixtures. The results show that the oxygen from air resides in the C₅-formyl group whereas the lactame group stems from water [64].

The results indicate the action of a mono-oxygenase in the primary ring-cleaving step. This enzyme inserts only one oxygen-atom of an oxygen molecule into the product. *In vivo* labeling experiments with ¹⁸O and *C. kessleri* has shown that the additional oxygen atom at position C₁₀ in **9** stems from molecular oxygen [55].

In a recent study, by *in vitro* assays comprising ¹⁸O₂ Pheophorbide a oxygenase and a red Chl catabolite (RCC) reductase from *Brassica napus* yielded an oxygen labeled fluorescent Chl catabolites. Only one oxygen label was found to reside in the formyl group of the catabolite. This result suggests that Chl

breakdown is mechanistically related to each other in higher plants and in green algae [65].

In contrast to the enzymatic oxidation, chemical photo-oxidation of the Cd(II) pyropheophorbide a methyl ester in presence of $^{18,18}\text{O}_2/^{16,16}\text{O}_2$ -mixtures has shown that two oxygen atoms are incorporated into the pigment. Both arise from the same oxygen molecule [66] indicating that the formation proceeds by a $[\pi 2 + \pi 2]$ cyclo-addition. On the other hand, in phycobiline biosynthesis and in heme catabolism, three different oxygen molecules are involved in the ring cleavage mechanism [42].

Terminal step of the primary ring cleavage

When first cultured under mixotrophical conditions in a medium containing heavy water (59 atom% ^2H), green cells of the Chlorophyte *C. protothecoides* excrete characteristically deuterium labeled red Chl catabolites into the culture medium (Fig 10) [67]. ^1H -NMR spectroscopic analysis of the methyl ester derivative revealed a highly stereo-controlled addition of the hydrogen atom to the former C10-methine group.

Albeit the absolute configuration at C10 of the labeled catabolite remains currently unknown, the results show that this step is most likely enzymatically controlled. Moreover, the integrity of the deuterium label in the formyl group, which originates from the former C5-methine group of the Chl macro-cycle, excludes the formation of a 5-oxophlorin intermediate. Otherwise, the formyl group would have exchanged the label.

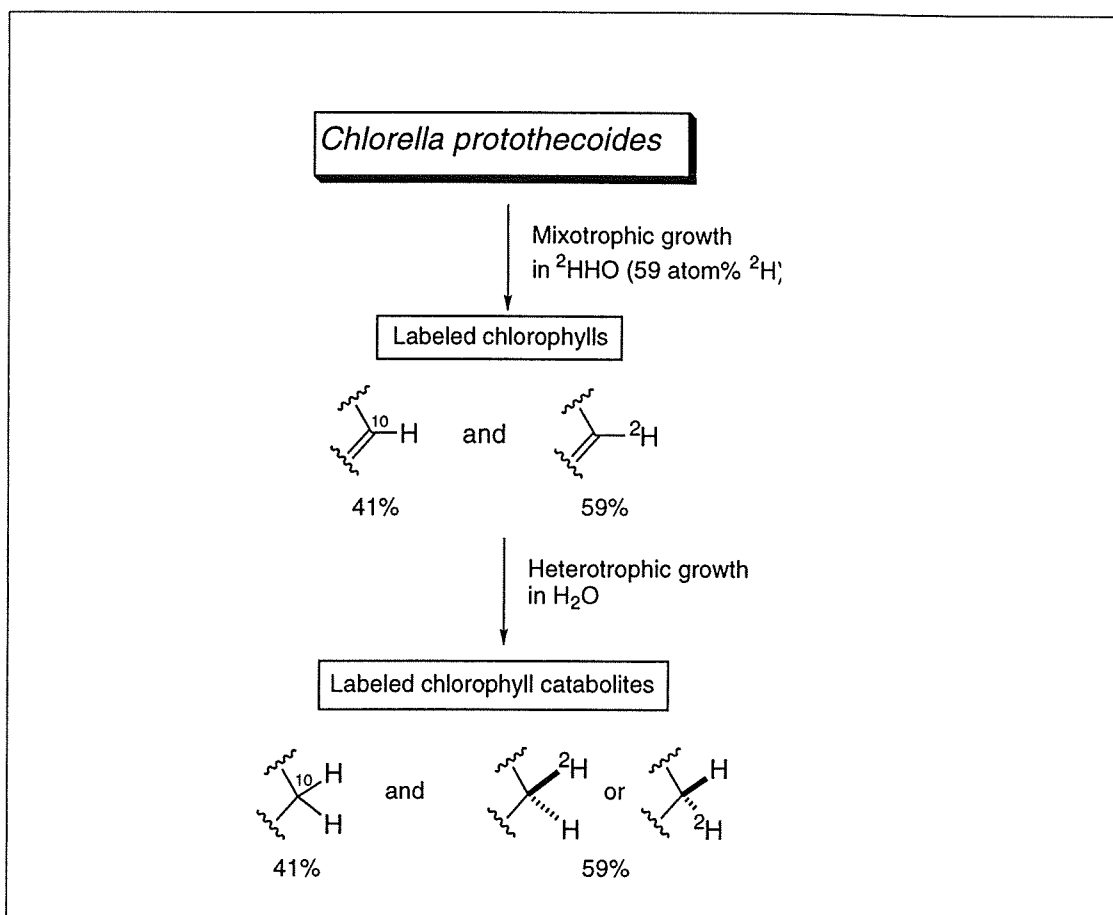


Fig. 10 *In vivo* labeling experiments with *C. protothecoides* in the presence of heavy water (59 atom%D). Exclusively one isotopic epimer has been detected, the absolute configuration of which is unknown[67].

Pyrroline to pyrrole-ring transformation

Acid catalyzed pyrroline to pyrrole ring rearrangement of the red pigment isolated from *C. protothecoides* was studied in detail (Fig. 11) [39]. The rearrangement proceeds in the presence of neat achiral acids such as acetic and propionic acid and is indicated by the loss of the red color. The final products are yellow, non-fluorescent pigments. A mechanism for this unusual reaction has been tentatively suggested and rationalized as a sequence of two consecutive imine-enamine tautomerisations. The reaction is highly enantioselective yielding an optically active product with an $ee = 98\% \pm 2$ for the *Z*-isomer **7a**. When the same reaction is carried out with the analogous *E*-isomer **8a** the same enantiomer is obtained as the major product although the

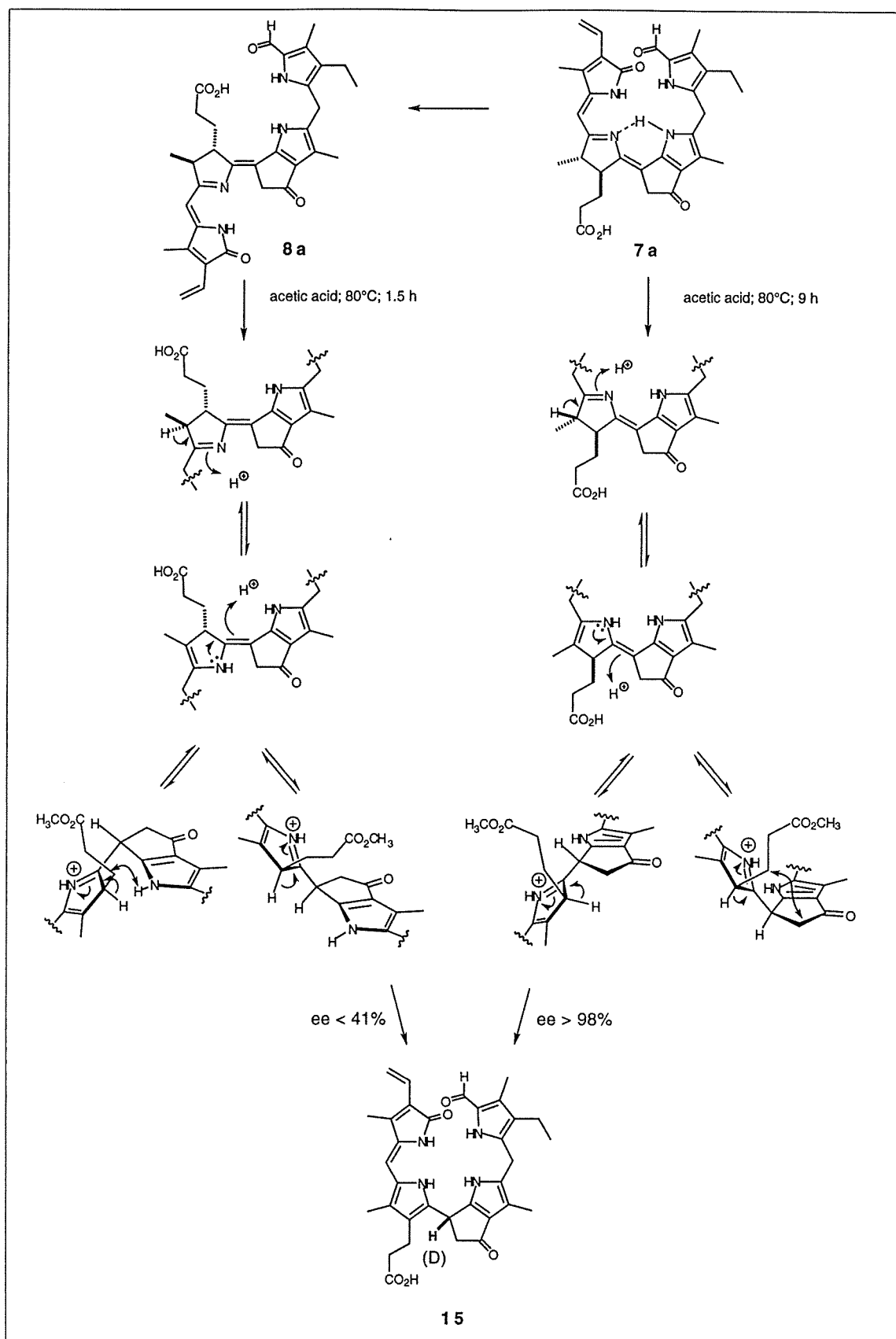


Fig. 11 Suggested reaction mechanism for the prototropic rearrangement of the red Chl a catabolite. Double-headed arrows indicate steric repulsion. Compound 15 is expected to be R configured[39,68].

stereoselectivity of the reaction is less pronounced: an ee = 77%±2 was observed.

The energy of activation of the reaction was determined in acetic acid by an Arrhenius plot between 60° and 110°C to $\Delta E = 76$ kJ/mole for the Z-isomer and $\Delta E = 74$ kJ/mole for the E-isomer [39]. In deuterated acetic acid the H-atom on the C15 position is not exchangeable by deuterium in the final product of the reaction but is incorporated during the transformation. It follows that the transformation proceeds by an intermolecular rearrangement. This mechanism would anticipate for stereochemical reasoning the R-configuration of the ligands at C15-carbon atom (Fig. 11) [39,68]. Unfortunately, a suitable crystal for X-ray crystallography to determine the absolute configuration of this center is not yet available. The transformation of the pyrroline ring into a pyrrole ring is accelerated when the number of conjugated C=C bonds decreases (*vide infra*).

Catalytic hydrogenation

Partial catalytic hydrogenation of the main red pigment of *C. protothecoides* affords two yellow fluorescent compounds (16) and (17) (Fig 12) as diastereomeric mixtures both exhibit a quantum yield at 423 nm of $\Phi = 1.8$ and $\Phi = 8.4$, respectively [39]. On subsequent treatment with acetic acid, compounds 19 and 20 are isolated as non-fluorescent colorless products.

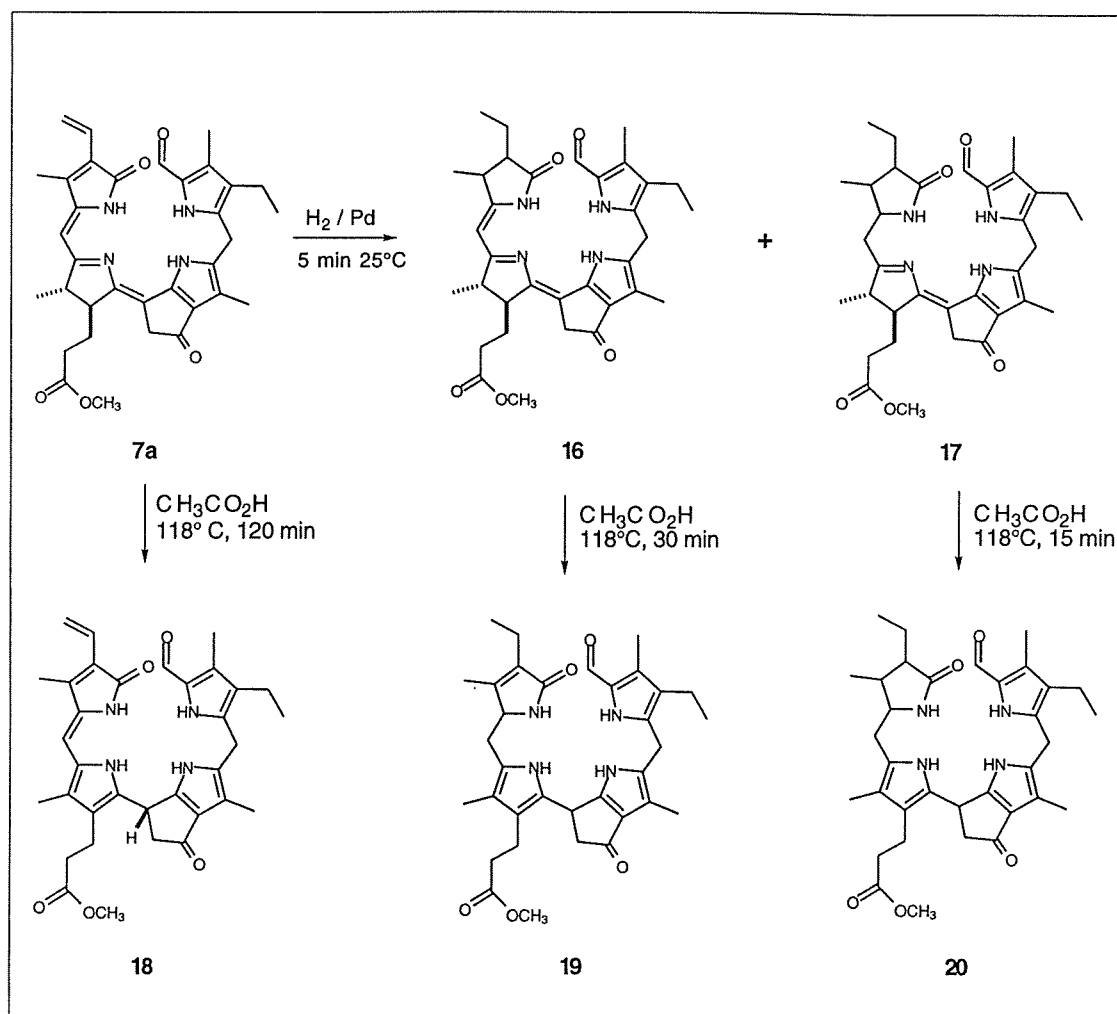


Fig. 12 Products of the pyrroline/pyrrole ring rearrangement of the red Chl catabolite **7a** and of its partially hydrogenated products **16** and **17**. The rate constant of conversion increases with the degree of saturation. The fluorescence, characteristic for the starting materials, disappears completely. During the rearrangement in presence of per-deuterated acetic acid, the C15-position is deuterium labeled; high enantioselectivity was observed using propionic acid. Compound **19** represents the structural skeleton of the catabolites isolated from higher plants (compare Fig. 7).

The more reduced the more the rearrangement is facilitated. From this result we anticipated that the enzymatic reduction of the $\text{C}_{20}=\text{C}_1$ bond in plants precedes the pyrroline/pyrrole-ring transformation. The migration of the exocyclic $\text{C}_{20}=\text{C}_1$ double-bond of **16** into the lactame ring corresponds to the well-documented acid-catalyzed rearrangement of bilirhodines into urobilines [69].

Enzymatic *ex vivo* systems

Fluorescence of the leaves during Chl degradation has been observed several times; the compounds responsible for fluorescence were regarded as precursors of the final plant catabolites [70]. This metabolite was traced down to the intact isolated senescent chloroplast. Consequently, an *ex vivo* degrading system was implemented in which isolated senescent thylakoid membranes from rape cotyledons were used [71]. Incubation of the latter with pheophorbide *a* as substrate afforded the fluorescent intermediate **21** which still contains an intact pyrroline ring system (Fig. 13). The system required dioxygen and several cofactors to keep externally added ferredoxin, a non-heme iron compound, in the reduced state.

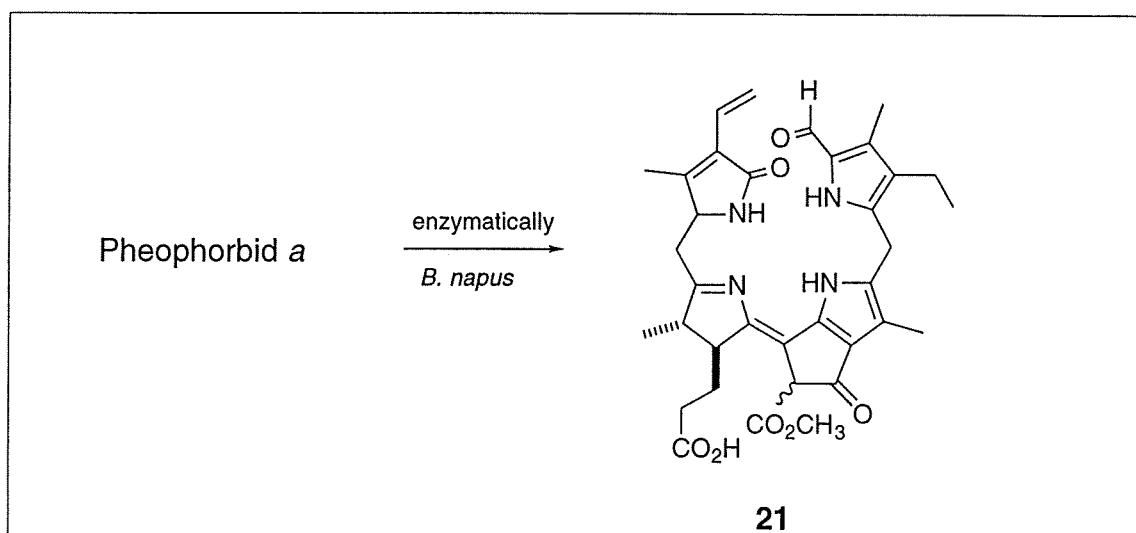


Fig. 13 Catabolite **21** was isolated from an incubation extracts of *Brassica napus* [71] and is regarded as a metabolic intermediate. **22** was detected after incubation of senescent chloroplast membranes of *B. napus* with pheophorbide *a* as substrate.

Most recently, it was demonstrated that the red Chl *a* catabolite **22** was converted into **21** by incubation with a stroma protein-preparation from barley [72]. The result showed that the reduction of the C₂₀=C₁ of the

δ -methine bridge precedes the pyrrolin-ring rearrangement. Notably, in the reaction product the C₁₃² methyl ester group remained intact whereas the C₈²- β -hydroxyl-group, characteristically for the final plant catabolites, was absent.

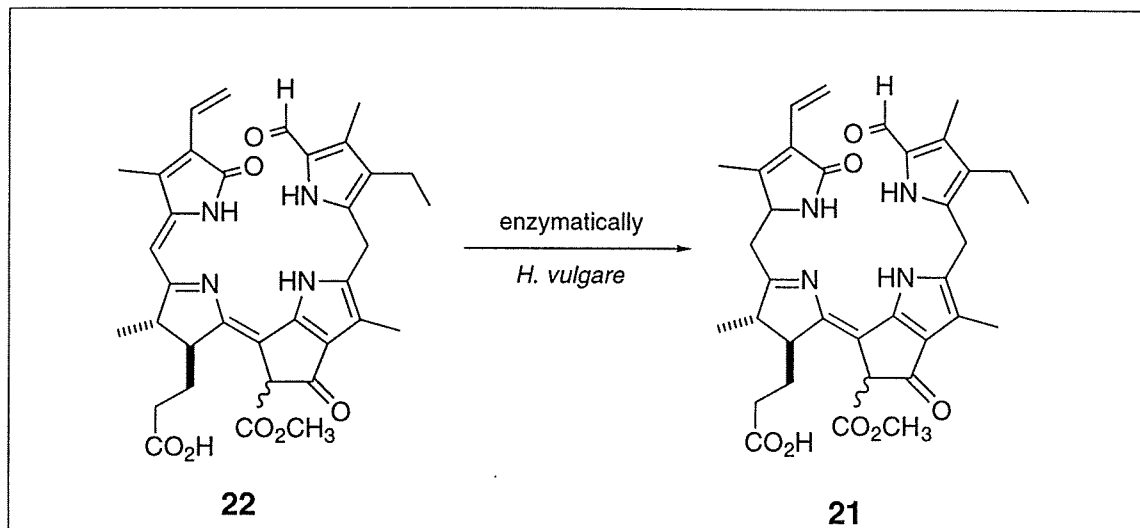


Fig. 14 Enzymatic conversion of 22 with a stroma protein from *H. vulgare* [72].

Chlorophyll *b* to chlorophyll *a* reductase

Although both Chl *a* and *b* catabolites were isolated from *C. protothecoides* [52,54], only catabolites of Chl *a* were isolated from vascular plants. As a matter of fact, Chl *b* disappears together with Chl *a* in higher plants. Further, pheophorbide *b* was not recognized as a substrate in the above-mentioned pheophorbide oxygenase reaction, instead was even found to be a competitive inhibitor of this enzyme [71]. Recently, an enzymatic system, able to convert Chl *b* in Chl *a*, has been detected in cucumber etioplasts of *Cucumis sativus* L [73]. From this result it has been suggested that in higher plants Chl *b* is transformed into Chl *a* via 7-hydroxymethyl derivative 23 prior to degradation (Fig 15). Based on this observation, lysed plastids of barley leaves were incubated with NADPH, and in an additional experiment with Zn-pheophorbide *b*. 7¹-hydroxy-chlorophyll *a*, 7¹-hydroxy-chlorophyllid *a* and Zn-7¹-hydroxy-chlorophyllid *a* were isolated from the incubation mixture indicating activity of a Chl(ide) *b* reductase [74].

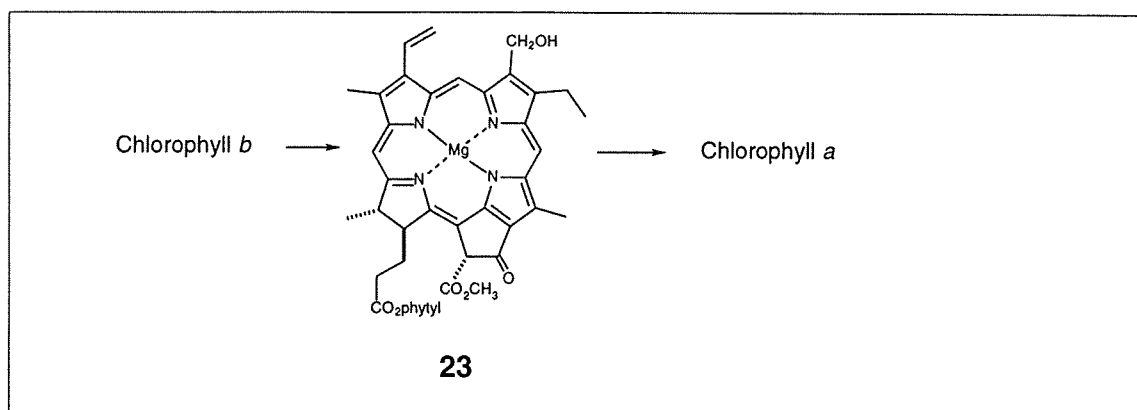


Fig. 15 In etioplasts of *Cucumis sativus*, Chl *b*-to-Chl *a* conversion proceeds via the 7-hydroxymethyl derivative **23** [73].

In order to prove this hypothesis *in vivo* deuterium isotopic labeling of the catabolite of barley (*Hordeum vulgare*) has been performed [75]. Therefore, green leaves of barley were de-greened in permanent darkness in presence of heavy water. The plant extract after a crude purification procedure was oxidized with diluted $\text{CrO}_3/\text{H}_2\text{SO}_4$ solution. The maleimide fragment, which contained the characteristic β -hydroxyethyl group of ring B (Fig. 7), was isolated and analyzed by spectroscopic methods. The data evinced that a fraction of the methyl group was deuterium labeled. This result suggests that Chl *b* is converted to Chl *a* before degradation. It was surprising to find that this fraction was exclusively mono-deuterium labeled, indicating that the reduction of the formyl group proceeds in two distinctive steps. In analogy to the co-factors known to be necessary for the *in vitro* reduction of Chl *b* to Chl *a* a mechanism was suggested. The first step was rationalized as a reduction of the formyl group by co-enzyme NAD(P)H. This yields the corresponding 7-hydroxymethyl derivative **23** containing two hydrogen atoms. The hydride of NAD(P)H must, therefore, be generated from a carbon-hydrogen bonds of (poly)saccharides biosynthesized in the protic environment of the greening phase of the plant; NAD(P)H is known not to exchange its protons or deuterons with the surrounding medium [76]. Subsequent reductive elimination of the hydroxyl group appears to be an electron transfer process.

Activation of the hydroxyl group by ATP and elimination would generate a carbenium-ion which is reduced by two electrons. The co-factor necessary in the *ex vivo* experiments for this process is ferredoxin able to donate electrons to the carbenium-ion. Quenching with a deuteron of the surrounding medium would then introduce one deuterium atom in the methyl group.

Green Algae as Progenitors of Higher Plant Cells

Fossil records indicate that most ancient organism which contain Chl *a* were probably blue-green algae dating back possibly 3 billion years into the Precambrian [77]. These organisms were followed by several groups of eukaryotic algae in the later Precambrian [78]. The antiquity of algae in the history of living organism argues for their primacy in the plant kingdom (Fig 16).

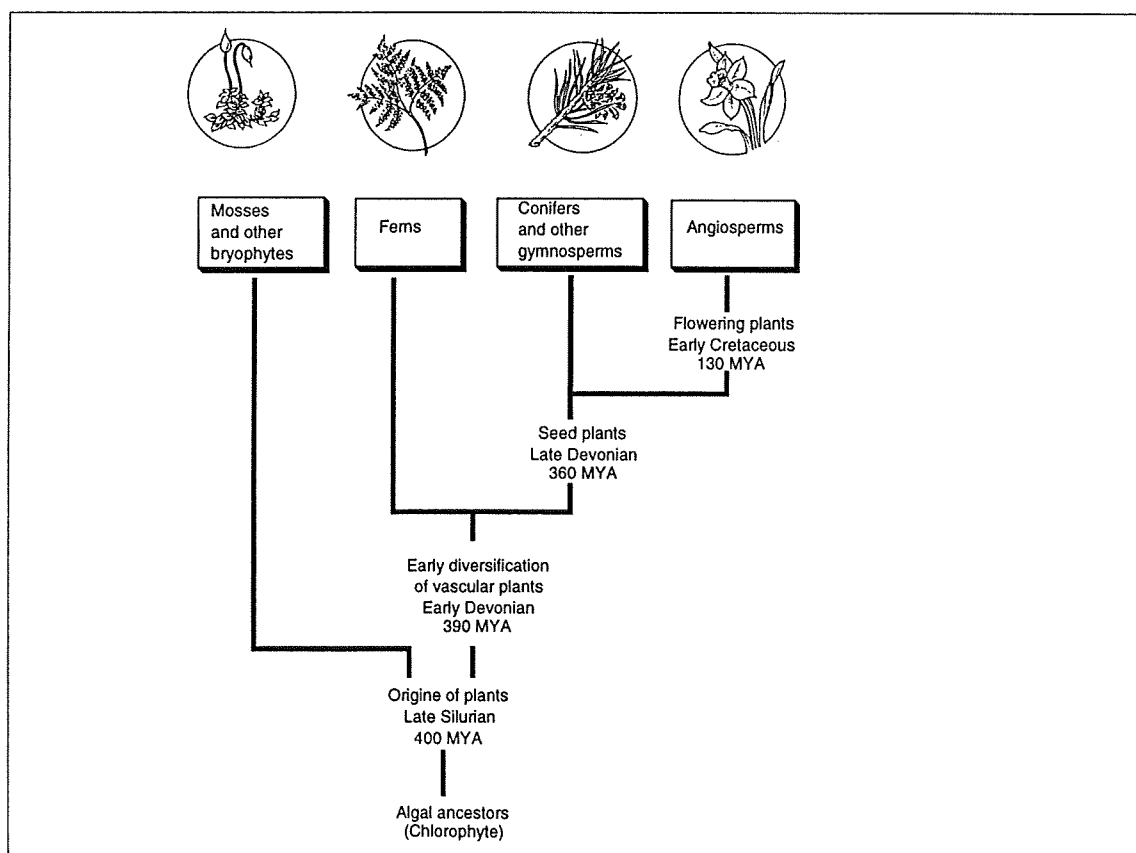


Fig. 16 Evolutionary relationship of green algae and higher plants, MYA: million years ago. (Adopted from Campbell, Biology, Cunningham) [79].

Most botanists today view algae, especially the green algae, as likely progenitors for the remaining members of the plant kingdom because they are similar in pigmentation having Chl *a* and *b* and starch in their storage reserves. Based on comparisons of nucleotide sequences in the 18S and 28S cytoplasmatic rRNAs an extraordinary intimate phylogenetic relationship of the phylum *Chlorophytes* to the phylum *Tacheophyta* was determined. This result confirms the hypothesis that the vascular plants evolved from a green algal ancestor [80]. Therefore, it was speculated that Chl catabolism is of very ancient evolutionary origin [81]. The occurrence of structurally similar catabolites isolated from different plant families supports this hypothesis, until now.

Tentative Degradation Pathway

Based on morphological, physiological and molecular biological similarities, biologists have generally accepted that the higher plant cell has evolved from a chlorophytic ancestor [79]. The similarity of the oxidative ring cleavage products of the chlorin macrocycle found in both chlorophytes and angiosperms indicates a fundamental catabolic process. The catabolites isolated from today's chlorophyte *C. protothecoides* appear to have preserved rudimentary structures when compared with the catabolites isolated from diverse higher plant families. Fig. 17 summarizes the catabolic pathway of the Chls based on current structural assignments, mechanistic investigations and chemical plausibility.

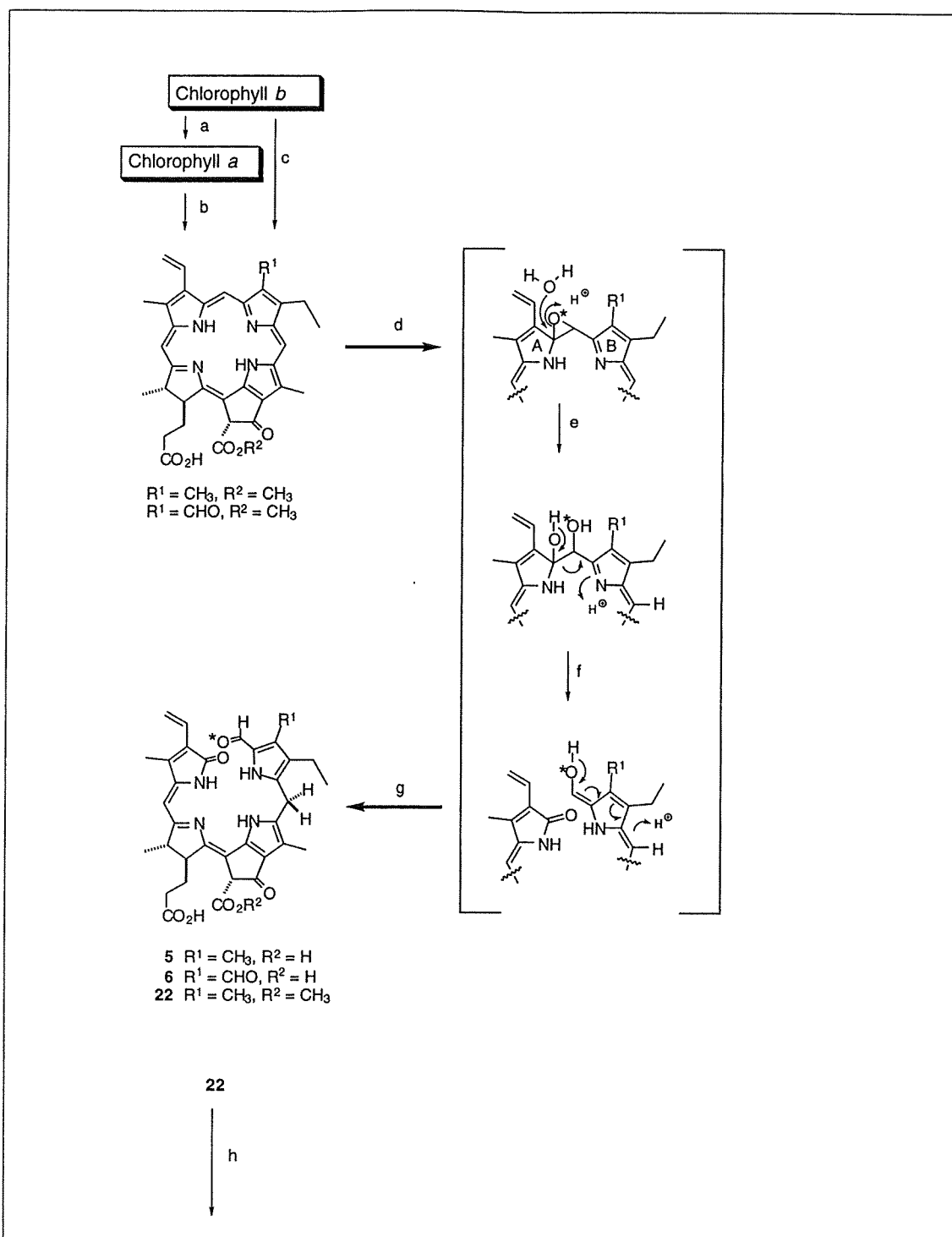


Fig. 17 Suggested catabolic pathway of the Chls in the green plant lineage. Mechanistic details are framed within dotted lines. The configuration of the methyl ester group at position C13² is consistently drawn as found in the Chls although this position is prone to epimerization. In contrast to earlier assumptions, light appears not to be necessary for the degradation of the Chls. Continues next page.

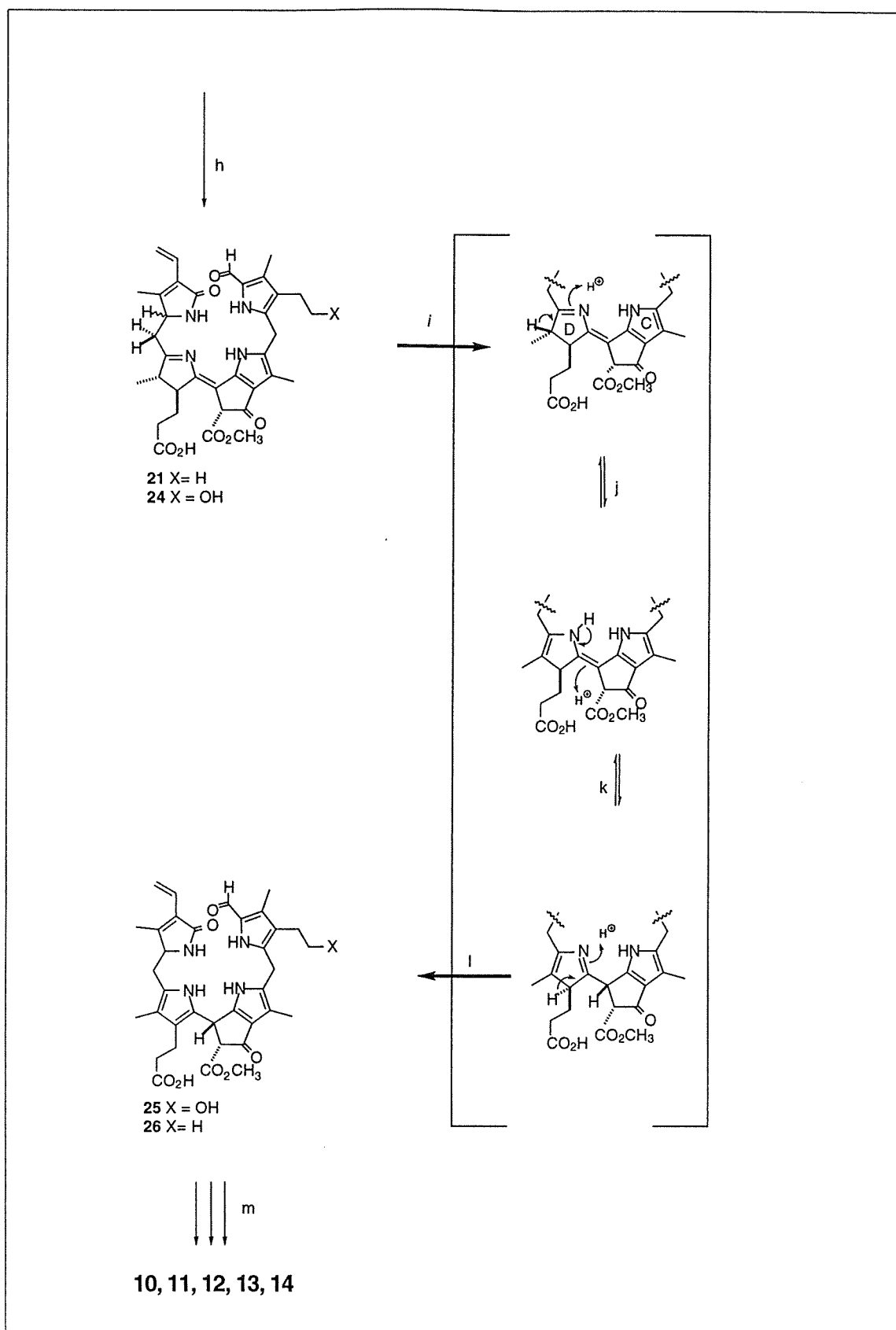


Fig 17 continuation

Key to Fig 17

a In angiosperms only catabolites arising from Chl *a* have been isolated, Chl *b* is converted to Chl *a* before degradation.

b Both enzymes, Chlorophyllase and magnesium dechelatase convert Chl *a* into pheophorbide *a*, the latter has been accepted as substrate in preparations made from *B. napus*.

c In contrast, the green alga *C. protothecoides* excretes both Chl *a* and *b* catabolites into the medium. The actual precursor of the primary cleaving step has not yet been determined.

d Regioselective addition of a monooxygenase-activated oxygen molecule to the C₄=C₅ bond; the oxygen atoms arising from molecular oxygen are differentiated by an asterisk.

e Regioselective cleavage of the oxirane ring by spontaneous or enzymatic hydrolysis in which the label remains bound to position C₅.

f Retro-aldol condensation cleaves the macrocycle.

g Enantioselective proton rearrangement eventually yields the isolated red Chl catabolites **5** and **6**; only one isotopic epimer has been detected. The sequence of methylester hydrolysis is unknown. Compound **22** is considered as short living intermediate in angiosperms.

h The hydrogenation of the C₂₀=C₁ bond by a reductase proceeds stereoselectively, the four stereogenic centers of **21** are uniformly configured.

i Pyrroline/pyrrole ring rearrangement starts by protonation of the imino group of the pyrroline ring, which subsequently rearranges to a corresponding enamine.

j Reversible enamino/imino rearrangement protonates position C₁₅ from both possible heterotopic faces.

k Of the both conceivable activated complexes one is favored because of lesser sterical interaction between the propionic side chain and the isocyclic ring. Both compounds **5a** and **6a** yield the same enantiomer when heated in acetic acid, the favored configuration of the product is assumed R.

l Aromatisation to the pyrrole ring moiety by prototrophic rearrangement yield **26**.

m oxidative β -hydroxylation of the ethyl group and further modification leads subsequently to the Chl *a* catabolites (**10-14**) isolated from angiosperms. Actually, the sequence of side chain modifications is not yet known. Hydroxylation and/or introduction of the glycol group, observed in the catabolites of *H. vulgare*, may occur before or after the pyrroline/pyrrole ring rearrangement.

FUTURE PROSPECTS

Molecular and mechanistic aspects of the Chl catabolism have received considerable attention during the last years. The present knowledge of this process suggests a consistent picture in which the evidence gained from taxonomically different organisms is until now in significant good agreement. Nevertheless, the catabolic pathway depicted in Fig. 17 should be regarded as a working hypothesis. Under consideration of phylogenetic relationships additional catabolites or their intermediates should be isolated and characterized in order to challenge the present mechanistic proposition.

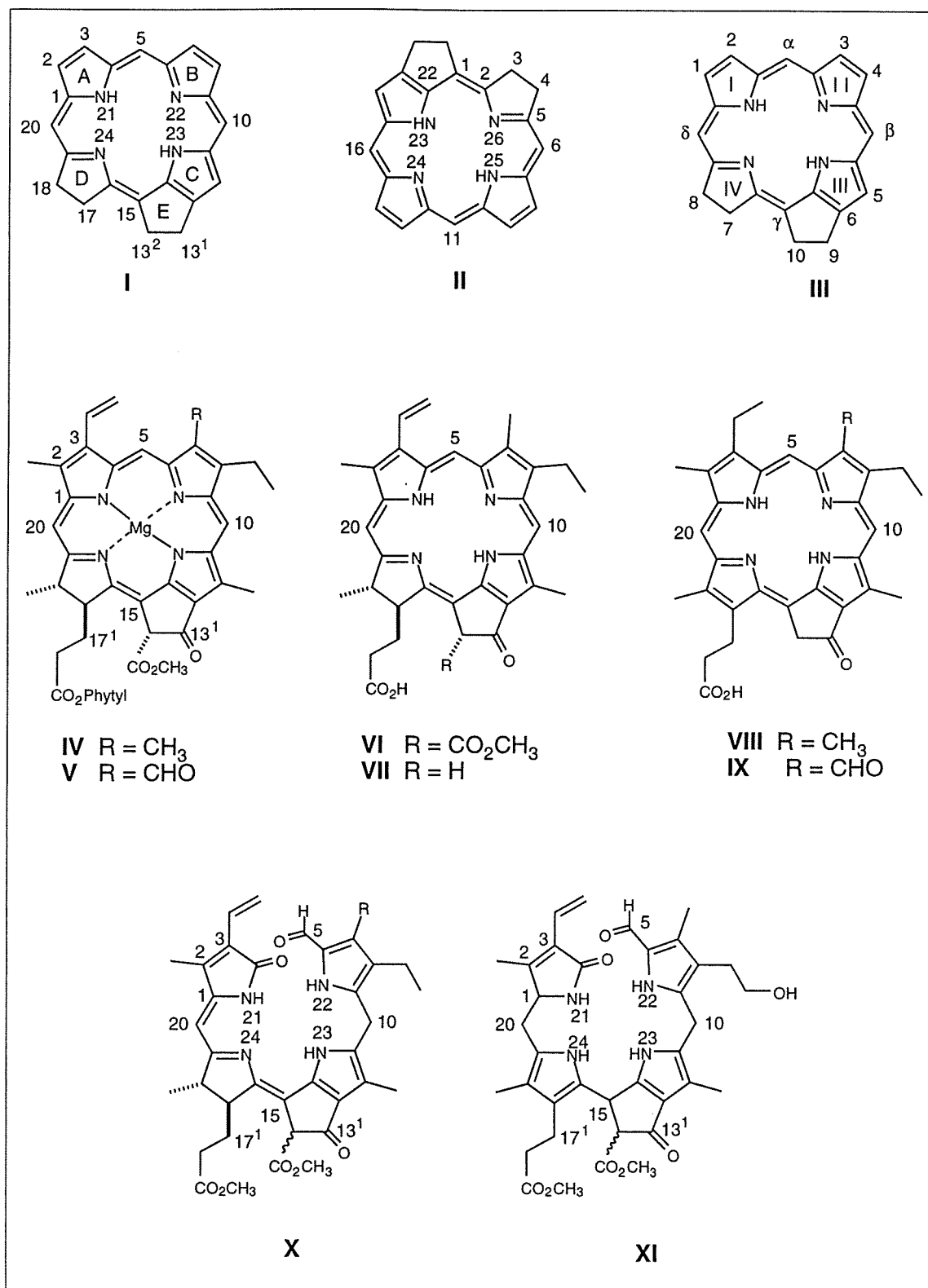
The sequence of reactions, the mechanisms and the stereochemical course during the Chl degradation need to be determined in detail. Catabolites from algae and higher plants consist of several chiral and prochiral centers that need to be determined, because homochirality in the stereogenic centers will show if a similar set of enzymes is active or not. Enzymatic crossover experiments and genetic analyses might further support the evolutionary relationship between green algae and higher plants and the ubiquity of the catabolic pathway of the Chls in green plant lineage. Investigations on the biological aspects of the degreening process and the cloning of degreening-related genes in *C. protothecoides* are currently beginning to evolve [82].

The deciphering of the catabolism of the Chls is currently a matter of basic research which impinges on several research areas such as chemistry, biochemistry, various aspects of plant biology, geochemistry and food processing. Finally, the catabolites itself represent a new class of natural compounds with unknown chemical- physico-chemical and physiological properties, which are attractive for further investigations.

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APPENDIX



Nomenclature of the Chls and of their degradation products [83-86].

First row: different numbering systems have been implemented for phorbine, the basic nucleus of the Chls. Formula I, shows the numbering system commonly in use for Chl and its derivatives according to IUPAC-IUB 1979. This convenient numbering system is applied throughout this text. II has been recommended by IUPAC-IUB since 1987. Formula III is being found in the older literature and was suggested by H. Fischer.

Second row: structure of Chl *a* IV and *b* V, pheophorbide *a* VI, pyropheophorbide *a* VII and phytylporphyrine *a* VIII and *b* IX. These formulae are used as templates to generate semisystematic names and numbering of the Chl catabolites.

Third row: ring cleavage products of the Chl macrocycle are denominated in the present work as *seco* derivatives according to IUPAC-IUB rule R-1.2.6.2. For example, the esterified Chl catabolite X isolated from *Chlorella protothecoides* is regarded as 10,22-dihydro-4,5-dioxo-4,5-*seco*[21H,23H]pheophorbide *a* methyl ester. The methyl ester derivative XI of the plant catabolite isolated from *Cercidiphyllum japonicum* is viewed as a phytylporphyrine *a* derivative and is denoted 3¹,3²-didehydro-1,10,15,20,22,24-hexahydro-8²-hydroxy-13²-methoxycarbonyl-4,5-dioxo-4,5-*seco*[21H,23H]phytylporphyrinate *a* dimethyl ester.

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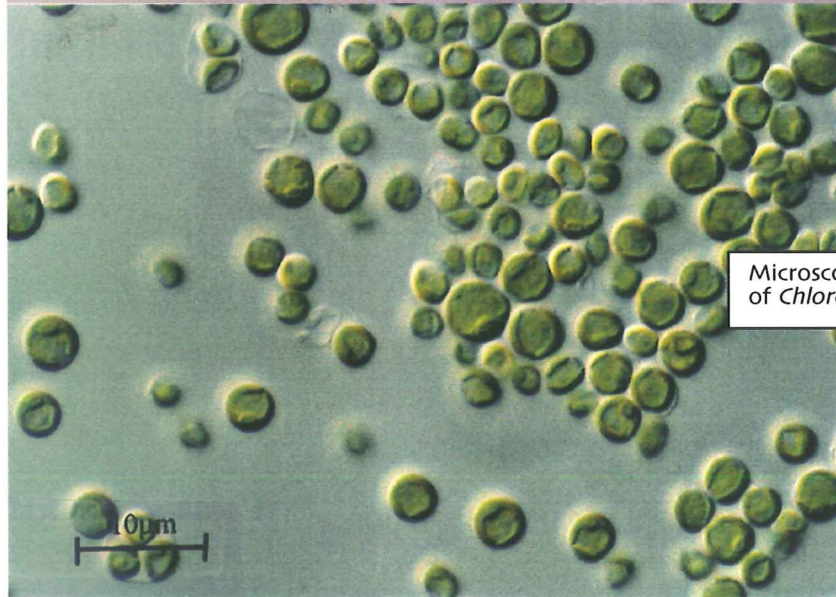
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Autumnal leaves on a branch of *Cercidiphyllum japonicum*



Cotyledones of barley (*Hordeum vulgare*) after 7d in permanent darkness. Left: leaves soaked in tap water; right: in heavy water (80 atom% deuterium)



Microscopic image of green cells of *Chlorella protothecoides*

Chlorophyll *b* to Chlorophyll *a* Conversion Precedes Chlorophyll Degradation in *Hordeum vulgare* L.*

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This study reveals by *in vivo* deuterium labeling that in higher plants chlorophyll (Chl) *b* is converted to Chl *a* before degradation. For this purpose, de-greening of excised green primary leaves of barley (*Hordeum vulgare*) was induced by permanent darkness in the presence of heavy water (80 atom % ²H). The resulting Chl *a* catabolite in the plant extract was subjected to chemical degradation by chromic acid. 3-(2-Hydroxyethyl)-4-methylmaleimide, the key fragment that originates from the Chl catabolite, was isolated. High resolution ¹H-, ²H-NMR and mass spectroscopy unequivocally demonstrates that a fraction of this maleimide fragment consists of a mono-deuterated methyl group. These results suggest that Chl *b* is converted into Chl *a* before degradation. Quantification proves that the initial ratio of Chl *a*:Chl *b* in the green plant is preserved to about 60–70% in the catabolite composition isolated from yellowing leaves. The incorporation of only one deuterium atom indicates the involvement of two distinguishable redox enzymes during the conversion.

Chl ¹*b* occurs as an accessory pigment of the light harvesting systems in higher plants, green algae, Euglenaceae and Prochlorophyta and comprises up to 30% of the total Chls (1). As a matter of fact, although both Chl *a* and *b* catabolites were found in the green alga *Chlorella protothecoides*, only catabolites originating from Chl *a* were isolated from higher plants (2, 3). The following observations have led to the hypothesis that in higher plants Chl *b* is converted into Chl *a* before degradation: (i) A system comprising isolated etioplasts of cucumber *Cucumis sativus* showed that chlorophyllide *b* is converted to Chl *a* (4). Subsequent studies of a system consisting of barley (*Hordeum vulgare*) etioplasts showed that chlorophyllide *b* is converted via 7¹-hydroxy Chl *a* to Chl *a*; both steps required the presence of ATP in the incubation mixture (5). (ii) *In vitro* chlorophyll degradation experiments with membrane fractions of senescent chloroplasts of rape cotyledons (*Brassica napus*) have shown that Pheo *b* is refused as substrate of the ring cleaving pheophorbide oxygenase under the condition that Pheo *a* is accepted (2). (iii) Zn(II) Pheo *b* was converted to Zn(II)

7¹-hydroxy Chl *a* in intact barley etioplasts, the reduction required NADPH or NADH. NADH was found to be less effective, ATP was not essential (6). (iv) Fully senescent cotyledons of rape (*B. napus*) contain amounts of Chl *a* catabolites 7a-c accounting for 90% of total Chls originally present in the mature leaf tissue (2, 7).

Chl *a* to Chl *b* conversion appears now to be part of a general Chl *a*(*b*) interconversion cycle, which is considered to play an important role in the formation and reorganization of the photosynthetic apparatuses (5) and which enables plants to adapt to high and low light conditions by adjusting the ratio Chl *a*:Chl *b* from 3.8–4.8 to 2.7–3.0, respectively (8).

In vivo labeling experiments with heavy water to elucidate biogenesis mechanisms are barely mentioned in the literature. The method has been applied to study peripheral changes of the porphyrin system during the biosynthesis of bacteriochlorophyll *a* in the photosynthetic bacterium *Rhodospirillum rubrum* (9, 10) and to follow the insertion of a deuteron during the light induced cyclization of dark synthesized acyclic carotenoid precursors in the green alga *Senedesmus obliquus* (11). Recently we have demonstrated by *in vivo* deuterium labeling experiments with *C. protothecoides* that in the last step of the macrocyclic ring cleavage a hydrogen atom is highly stereoselectively inserted in the catabolite (12).

In this work we evince by spectroscopic methods that during the de-greening of barley leaves (*H. vulgare*) in heavy water a fraction of the Chl *a* catabolite is deuterium labeled; specifically one deuterium atom is incorporated in the methyl group of the apparent chlorophyll *a* catabolite. The results suggest that Chl *b* is converted to Chl *a* by two different cofactors.

EXPERIMENTAL PROCEDURES

Chemicals and Materials—All chemicals were reagent grade; all solvents were distilled before use. Heavy water containing 80 atom % deuterium was supplied from Armair AG, CH-5312 Döttingen (Switzerland). Thin layer chromatography aluminum foils pre-coated with silica gel 60PF₂₅₄–366 (0.2 mm) and silica gel 60PF (0.040–0.063 mm) for column chromatography were purchased from Merck, Darmstadt, Germany and 35cc Sep-Pack[®] Vac C-18, 10 g from Waters (Milford, MA).

Plant Material—Barley seeds (*H. vulgare* L. cv. Gerbel) were a gift from Florimond Desprez, Cappelle-en-Pévèle 59242 Templeuve, France. The seeds were germinated in high density (5 seeds/cm²) in moist garden soil, purchased from a local market, and grown for about 7–10 days in natural light until the primary leaves reached about 10–15 cm in height.

NMR—¹H-, ¹³C-, and ²H-NMR measurements were performed on a Bruker Avance DRX-500 spectrometer operating at the frequencies of 500.13, 125.75, or 76.77 MHz, respectively. Samples were dissolved in CDCl₃. Chemical shifts were recorded in ppm downfield from tetramethylsilane except for deuterium NMR in which CDCl₃, δ = 7.27 ppm was used as internal standard. ¹H-Spectra for the labeled material were recorded at a resolution of 7.63·10⁵ ppm/data point. Gaussian multiplication of the free induction decay (13, 14) was performed on the NMR-Unix station with UXNMR version 2.1. (Parameters used: Gaussian broadening = 0.5 Hz, line broadening = –0.55 Hz).

Mass Spectroscopy (MS)—Mass spectra were obtained with a Bruker FTMS 4.7T BioAPEXII, using chemical ionization (CI) or electrospray ionization techniques in the positive mode. Electrospray ionization

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¹ The abbreviations used are: Chl, chlorophyll; Chlid, chlorophyllide; Pheo, pheophorbide; AcOEt, ethyl acetate; AcMe, acetone; AcOH, acetic acid; CH₂Cl₂, dichloromethane; EtOH, ethanol; MeOH, methanol; *d_n*, number of attached deuterium or hydrogen atoms in a molecule; ppm, parts per million; MS, mass spectroscopy; amu, atomic mass units.

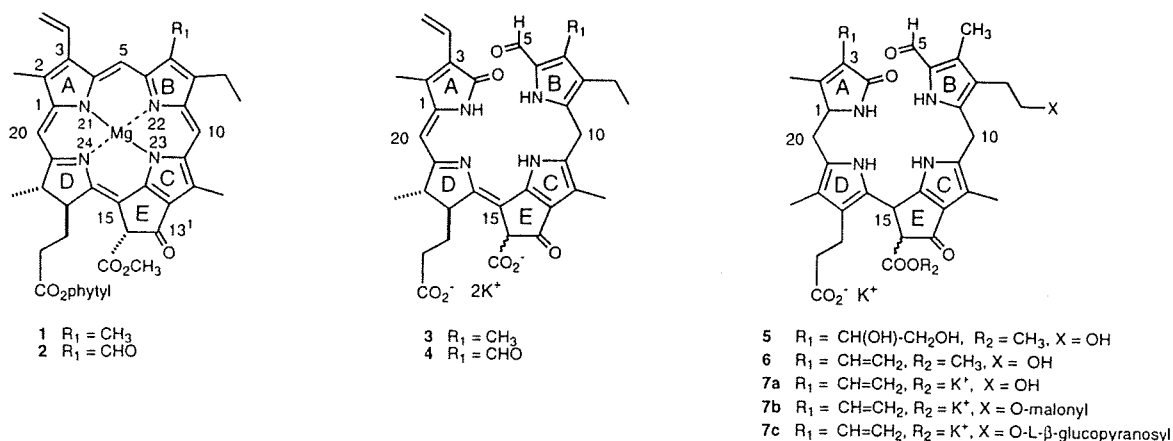


FIG. 1. Formulae of Chl catabolites isolated from green plants. *Chlorella protothecoides* 3, 4 (20, 21), *Hordeum vulgare* 5 (16), *Liquidambar spec.* (22) and *Cercidiphyllum japonicum* 6 (3), and *Brassica napus* 7a-c (7, 23). The structures 1 for Chl *a* and 2 for Chl *b* show the numbering system commonly in use for chlorophyll and its derivatives according to IUPAC-IUB 1979 (24, 25). This convenient numbering system is likewise applied to the ring cleavage products of the chlorophyll macrocycle, which are denominated as *seco* derivatives in accordance to IUPAC-IUB rule R-1.2.6.2. (26, 27).

spectra were expanded in the range of the molecular ion up to a resolution of 150,000. The most recent IUPAC data for atomic mass and natural abundance of the elements were used to calculate exact molecular masses (15).

De-greening of Primary Leaves of Barley—The procedure was similar as described previously (16) but with the following modifications: To arrest chlorophyll biosynthesis the green intact shoots were left at 25 °C for 12 h in permanent darkness. Afterward, batches of green primary leaves (100 g wet weight; 12 g dry weight) were cut 10–15 cm from the apex and immersed with their ends in heavy water (100 ml, 80 atom % ^2H). The opening of the 1,000 ml beakers was covered with punctured aluminum foil to allow gas exchange. The leaves were subsequently incubated at 25 °C during 7–8 days in permanent darkness. When the green color of the Chls had vanished, the yellowish still turgid leaves were collected and stored frozen until use. Unlabeled natural material was obtained accordingly using tap water instead of heavy water.

Determination of the Atomic $^1\text{H}/^2\text{H}$ Composition of Water—The watering layer was sporadically sampled, and the deuterium content of the water was determined by standard ^1H -NMR (360 MHz) procedure in which the remaining proton signal in the samples was measured. A sealed capillary filled with acetone was used as external proton standard whose signal area was determined from the area of pure water (H_2O) set to 100 atom % ^1H ; a relaxation delay of 60 s was applied during measurements to the sample.

Isolation of 3-(2-Hydroxyethyl)-4-Methylmaleimide (9) from Yellow Leaves of *H. vulgare*—De-greened yellow leaves of *H. vulgare* (150 g wet weight) were homogenized in a blender with a mixture of 0.1 M potassium phosphate buffer, pH 6.8:AcMe:MeOH = 1:1:1 (300 ml). The resultant slurry was filtered over two layers of cotton gauze, the residue was washed with the disintegration buffer (2×150 ml). The collected filtrates were centrifuged at $5,000 \times g_{av}$ for 10 min. Pellets were discarded and the supernatants were extracted with CH_2Cl_2 (2×400 ml). After phase separation the aqueous layer was shortly evaporated *in vacuo* to remove residual solvent. The remaining solution (200 ml) was filtered through a reversed phase column (35cc Sep-Pack® Vac C-18 cartridges). Afterward the cartridge was washed with 0.1 M potassium phosphate buffer, pH 6.8 (50 ml) and eluted with a 0.1 M potassium phosphate buffer, pH 6.8:AcMe = 1:1 (150 ml) solution. Retention and elution of the catabolite in the cartridge was controlled by a microscale chromic acid degradation assay using aliquots of the eluate (*vide infra*). Positive reacting fractions were pooled (120 ml), the volatile organic solvent was withdrawn *in vacuo* leaving a dark brown aqueous phase (60 ml). A solution consisting of $2 \times \text{H}_2\text{SO}_4$ and 1% CrO_3 (60 ml) was added with stirring at room temperature for 5 min. The resulting solution was continuously extracted overnight with diethyl ether. The sodium sulfate dried ether phase was evaporated to incipient dryness, and the residue was applied to four TLC plates. The plates were developed in $\text{CH}_2\text{Cl}_2/\text{AcOEt}/\text{EtOH}/\text{AcOH}$ (50:10:5:0.5). The section of the TLC foil containing maleimide fragment 9 was cut out and eluted with methanol (50 ml). Evaporation of the filtrate *in vacuo* left a residue, which was two times purified by microcolumn chromatography ($\varnothing = 4$ mm, length = 10 cm) using silica gel as stationary and CH_2Cl_2 /acetone

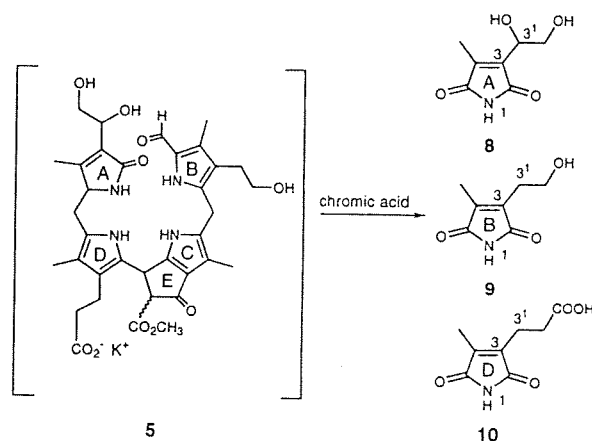


FIG. 2. Isolated maleimides 8, 9, and 10 obtained after CrO_3 oxidation of an extract from de-greened leaves of *H. vulgare*. Of the three fragments analyzed by ^1H -NMR only a fraction of the methyl group of 9 was deuterium labeled. The constitution of the Chl *a* catabolite from *H. vulgare* 5 (16) is shown in brackets (not isolated).

(8:2) as mobile phase. *Vacuo* evaporation of the effluent afforded 1.7 mg of pure maleimide 9. Accordingly, maleimides 8 and 10 were isolated from the CrO_3 oxidation mixture, data not shown.

Synthetic 3-(2-Hydroxyethyl)-4-Methylmaleimide (9)—Synthetic material was isolated as CrO_3 oxidation product of 3-(2-hydroxyethyl)-4-methyl-pyrrole. The pyrrole was synthesized in two steps² starting from benzyl-3-methyl-4-methoxycarbonylmethyl-5-methoxycarbonyl-2-pyrrolicarboxylate (18). The melting point of 9 is 104–109 °C. ^1H -NMR: 2.01 (t, $J = 0.9$ Hz, 3H, $\text{H}_3\text{C}(4^1)$), 2.67 (tq, $J_1 = 6.1$ Hz, $J_2 = 0.9$ Hz, 2H, $\text{H}_2\text{C}(3^1)$), 3.82 (t, $J = 6.1$ Hz, 2H, $\text{H}_2\text{C}(3^2)$), 7.17 (br. 1H, HN(1)). ^{13}C -NMR: 8.82 ($\text{C}4^1$), 27.53 ($\text{C}3^1$), 60.54 ($\text{C}3^2$), 139.20 + 140.49 ($\text{C}4 - \text{C}3$), 171.25 + 171.96 ($2 \times \text{C}=\text{O}$). MS-Cl: 156 ($[\text{M} + \text{H}]^+$, 100%), 138 ($[\text{M} - \text{H} - \text{H}_2\text{O}]^+$, 58%), 125 ($[\text{M} + \text{H} - \text{CH}_2\text{OH}]^+$, 6%). IR (KBr): 3,398 (very strong), 1,712 (very strong), 1,360 (strong), 1,086 (strong), 1,041 (strong), 738 (strong).

Maleimide Assay—200- μl samples were agitated with 200 μl of ether Et_2O and 200 μl of chromic acid solution (*vide supra*). The ether solution was spotted on thin layer chromatography and developed in $\text{CH}_2\text{Cl}_2/\text{AcMe}$ (8:2); $R_f(9) = 0.27$. The maleimide fragments were visualized with Cl_2 /benzidine (19).

RESULTS

General Description—Fig. 1 shows the constitutional formulae of the catabolites isolated so far and reveals that they are

² P. Folly and N. Engel, unpublished results.

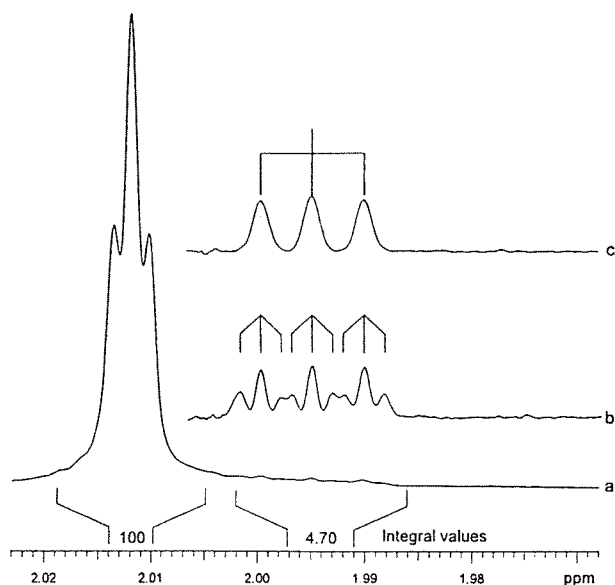


FIG. 3. 500 MHz proton spectrum of the maleimide fragment **9** isolated from *H. vulgare* after de-greening in heavy water (80 atom % ^2H). Only the region of the methyl group is shown. *a*, this trace shows the long range J_5 triplet coupling of the tri-protio-methyl group with the methylene group situated at $\text{C}(3^1)$. *b*, Gaussian multiplication of the free induction decay before Fourier transformation and amplification of the signals in close proximity to the tri-protio-methyl group unveils the fraction of molecules having a geminal $^1\text{H}/^2\text{H}$ coupling. *c*, result of a double resonance experiment in which the methylene group $\text{C}(3^1)$ at $\delta = 2.67$ ppm was irradiated. The absence of a quintet in the up-field region excludes the presence of a di-deutero-methyl group.

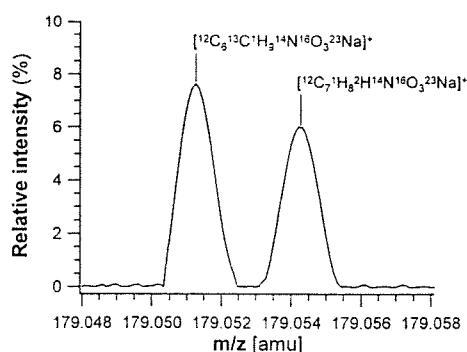


FIG. 4. Electrospray ionization-MS spectrum of the labeled maleimide fragment **9**. The spectrum was recorded on an ion cyclotron resonance instrument. The integral of the molecular peak ion at m/z 178.047, not shown, was set 100 (see Table I). Separation of the signals down to the base line was achieved at a resolution of 150,000. Carbon-13 fraction contains, in addition, the isotopomer with oxygen-17 together the signal integrates to an area of 7.7 rel %.

all bile-pigment-like linear tetrapyrroles derived from an oxygenolytic cleavage at the $\text{C}(4)=\text{C}(5)$ bondage of the former Chls. De-greening experiments were performed with green primary leaves of *H. vulgare* in heavy water (80 atom % ^2H) or in tap water, respectively. The crude enriched plant extract was directly subjected to the chromic acid degradation procedure basically developed for porphyrins (19, 28). The particular maleimide fragment 3-(2-hydroxyethyl)-4-methyl-maleimide (**9**) (Fig. 2), which contains the characteristic β -hydroxyethyl as marker group of the former Chl *a* catabolite of *H. vulgare*, was isolated and spectroscopically analyzed. ^1H - and ^{13}C -NMR and mass spectra of the synthetic and the unlabeled natural material were alike and in accordance with structure **9**. All seven carbon-bound protons were assigned by chemical shift reasoning, coupling constants, and integral values (see "Experimental Procedures"). The N-bound hydrogen atom occurred as single broad signal and the O-bound proton was part of the water peak of the solvent.

Analysis by Nuclear Magnetic Resonance Spectroscopy—The maleimide fragment **9** isolated from the labeling experiment of *H. vulgare* shows a small additional signal group in close proximity to the methyl group $\text{C}(4^1)$ at $\delta = 2.012$ ppm.³ After Gaussian multiplication of the free induction decay and amplification this signal group unambiguously displays the resonance pattern of a mono-deuterated methyl group (Fig. 3). The resonance is centered at $\delta = 1.995$ ppm and appears as a characteristic triplet \times triplet. In a double resonance experiment in which the methylene group $\text{C}(3^1)$ at $\delta = 2.67$ ppm was irradiated, the triplet of the tri-protio-methyl group collapsed as expected into a singlet and the signal group into a simple triplet with a coupling constant of $J_{\text{H}-^2\text{H}} = 2.4$ Hz in a ratio of 1:1:1 (Fig. 3). Both, spin multiplicity and signal ratio immediately evince due to the nuclear spin quantum number $I = 1$ of deuterium that the number of deuterium atoms in the methyl group equals 1. The mono-deuterated methyl group is up-field shifted by $\Delta\delta = 0.017$ ppm relative to the tri-protio-methyl group. This effect is within the range commonly observed for primary isotopic effects (29, 30). Integration of the signal of the tri-protio-methyl group versus the mono-deutero-methyl group revealed a proton ratio of about 100:4.7. From this figure a ratio of 9:9- d_1 of 100:7.1 was calculated. The ^2H -NMR spectrum confirmed the presence of one deuterium atom by a triplet ($J_{\text{H}-^2\text{H}} = 2.4$ Hz) centered at $\delta = 2.1$ ppm; no other signals were present except the solvent peak (spectrum not shown).

Analysis by Mass Spectroscopy—Samples were measured in an ion cyclotron resonance spectrometer equipped with an electrospray ionization inlet. Fig. 4 shows the isotopic fine struc-

³ The number of significant figures (4 sf) are not intended to indicate the accuracy of an absolute δ value but serves to calculate chemical shifts differences.

TABLE I

Calculated and measured mass spectroscopic data of the unlabeled and labeled maleimide **9** in the range of the nominal masses 178 and 179

Item	Nominal mass	Possible combinations of isotopic ions	Unlabeled (calculated) ^a		Labeled (measured)	
			Mass exact	Relative natural abundance	Mass exact	Relative abundance
	m/z (amu)		m/z (amu)		m/z (amu)	
1	178	$^{12}\text{C}_9\text{H}_9\text{N}^{14}\text{N}^{16}\text{O}_3\text{Na}^+$	178.04755	100	178.047 ^b	100
2	179	$^{12}\text{C}_7\text{H}_9\text{H}^{15}\text{N}^{16}\text{O}_3\text{Na}^+$	179.04449	0.4	— ^c	—
3	179	$^{12}\text{C}_6\text{H}_9\text{H}^{13}\text{C}^{14}\text{N}^{16}\text{O}_3\text{Na}^+$	179.05081	7.6	179.051 ^d	7.7
4	179	$^{12}\text{C}_7\text{H}_9\text{H}^{14}\text{N}^{16}\text{O}_3\text{Na}^+$	179.05167	0.1	—	—
5	179	$^{12}\text{C}_7\text{H}_8\text{H}^{15}\text{N}^{16}\text{O}_3\text{Na}^+$	179.05365	0.1	179.054	6.2

^a Isotopic mass and natural abundance of the constituting elements were taken from the literature (15). For the sake of correctness $0.55 \cdot 10^{-3}$ amu (rest mass of the electron) were subtracted from the uncharged molecular mass ions.

^b The scale was adjusted to the theoretical m/z value of the exact molecular mass ion.

^c Signal hidden in the noise.

^d The signals of the isotopomers containing carbon-13 or oxygen-17 are unresolved.

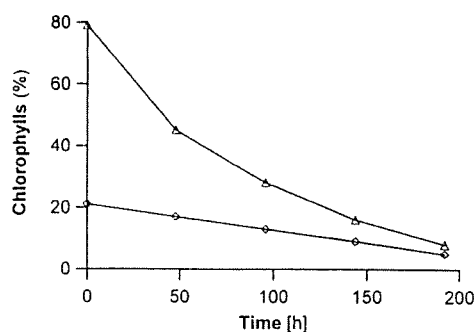


FIG. 5. Decrease of Chl *a* (Δ) and Chl *b* (\diamond) during the de-greening of barley leaves (*H. vulgare*) in presence of (heavy) water. Both pigments were separated by standard high pressure liquid chromatography techniques in which methanol extracts of the leaves were applied to a RP₁₈ column. The ratio Chl *a* to Chl *b* was determined with the means of a built-in UV-visible diode-array-detector working at the wavelength of 430 and 450 nm, respectively.

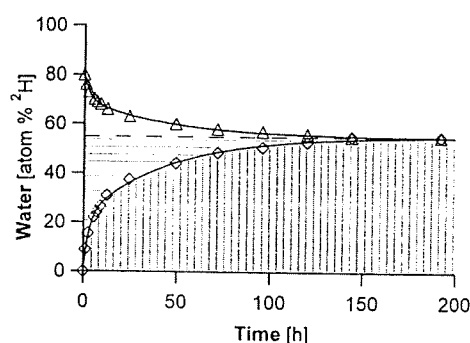


FIG. 6. Exchange rate between the leaves and the heavy water reservoir. Upper curve, time dependent dilution of the heavy water reservoir due to leaking of ordinary water from the leaves. An equilibrium concentration of 55 atom % ^2H was reached after about 192 h. Lower curve, the deuterium content in the leaves was calculated from the deuterium content of the watering layer and the equilibrium concentration. The vertically hatched area when subtracted from the total area limited by the dotted equilibrium line furnishes the horizontal hatched area, which represents the total amount of water (H_2O) in the leaves during the de-greening period.

ture at a resolving power of 150,000. The observed molecular peak ion $[\text{C}_7^{12}\text{H}_9^{14}\text{N}^{16}\text{O}_3^{23}\text{Na}]^+$ was calibrated to m/z 178.048 and an integral value of 100. Table I opposes the calculated and measured values. The signals are base line separated exhibiting the expected mass difference of 3×10^{-3} amu. The ion at 179.051 amu $[\text{C}_6^{12}\text{H}_9^{13}\text{C}^{14}\text{N}^{16}\text{O}_3^{23}\text{Na}]^+$ contains carbon-13 and shows the predicted abundance of 7×1.10 rel %. The second satellite signal at 179.054 amu $[\text{C}_7^{12}\text{H}_8^2\text{H}^{14}\text{N}^{16}\text{O}_3^{23}\text{Na}]^+$ contains ^2H and presents the number of molecules in which one protium is replaced by one deuterium. This fraction occurs with an abundance of 6.2 rel % from which 0.1 rel % was subtracted because of the natural abundance of deuterium of 9×0.015 . The molar ratio of 9:9- d_1 is therefore 100:6.1.

Quantitative Estimation of the Labeling Process—The ratio of Chl *a*:Chl *b* in the leaves during de-greening was followed by high pressure liquid chromatography of a methanol extract (Fig. 5). At the beginning of the incubation experiment this ratio was determined to be 79:21 (100:26). After 8 days in permanent darkness the incubation was terminated. About 10% Chl *a* and 24% Chl *b* of the initial content still remained unchanged in the leaves, which means that about 90% Chl *a* and 76% Chl *b* had vanished. Under the assumption that both Chls are totally converted into catabolites, a ratio Chl *a*:Chl *b* of 81:19 (100:23) should be expected. Random labeling of Chl *b*

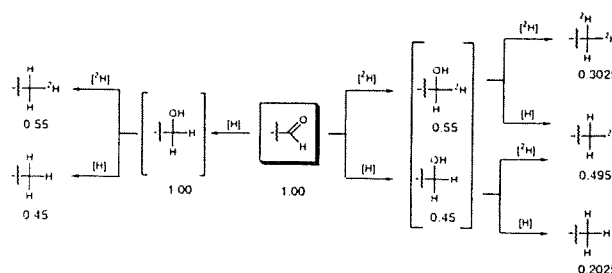


FIG. 7. Calculated deuterium label distribution pattern for a two-step reduction of a formyl (Chl *b*) into a methyl group (Chl *a*) in the presence of heavy water. During the incubation period the concentration in the watering layer diminished from 80 atom % ^2H to an equilibrium mixture of 55 atom % ^2H (see Fig. 6). Right, statistical [$^1\text{H}/^2\text{H}$] distribution over both steps. Left, selective reduction of the formyl group by [^1H] to the corresponding alcohol followed by statistical [$^1\text{H}/^2\text{H}$] labeling in the methyl group formation. The numbers indicate the fraction of moles of individual species carrying none, one, and two deuterium label(s).

in a medium of water containing 80 atom % ^2H should yield 80% mono-deuterium labeled Chl *a* and 20% of a "protio-labeled" Chl *a* catabolite in a ratio 9:9- d_1 of 100:17.6. As a matter of fact, the deuterium content of the heavy water in the beaker slowly diminished during the incubation period from 80 atom % ^2H to an equilibrium concentration of 55 atom % ^2H due to the dilution with protic water contained in the leaves (Fig. 6). Under the assumption that the number of deuterium atoms and the initial volume of 100 ml of water remains constant during the experiment the deuterium content of the leaves at each sampling point was calculated from the deuterium concentration of the watering layer and the equilibrium concentration by Eq. 1.

$$x_t = \frac{(x_{t=0} - x_{t=s})}{(x_{t=0} - x_{\text{equ}})} \cdot x_{\text{equ}} \quad (\text{Eq. 1})$$

where x_t = atom % ^2H in the leaves at the sampling time; $x_{t=0}$ = atom % ^2H in the water layer at zero time (80%); $x_{t=s}$ = atom % ^2H in the water layer at sampling time; x_{equ} = atom % ^2H in the water layer at equilibrium (55%).

The area under the calculated curve, which presents the time course of the relative amounts of 55 atom % ^2H in the leaves, was subtracted from the total area given by the square of the equilibrium concentration (55 atom % ^2H) and the incubation time (192 h). The difference assigns the total of the relative portion of pure water (H_2O) in mixture with 55 atom % ^2H in the leaves due to the slow exchange rate. This portion (13.6%) together with the protic fraction of the equilibrium mixture (45%) converts Chl *b* into the nonlabeled apparent Chl *a* in an amount of 1.4 and 7.7 rel %, respectively; the sum was therefore subtracted from the portion of Chl *b* and surcharged to Chl *a*. A theoretical ratio of 9:9- d_1 of 100:9.5 was calculated from those figures. This result is in close agreement with the spectroscopic measurements of a 9:9- d_1 relation of 100:6.1 for MS and 100:7.1 for ^1H -NMR and accounts for 64 and 74%, respectively, of the theoretical value.

The overall yield was calculated as follows. According to the literature, 115 g *H. vulgare* leaves afforded 13 mg of the intact catabolite **5** which represents a recovery rate of 10% of the original Chls (16). Quantitative conversion with CrO_3 oxidation to the maleimide fragment **9** should therefore yield 3.7 mg per 150 g leaves. In this labeling experiment 1.7 mg of purified **9** was isolated from 150 g leaves, which represents a yield of 46%. This loss is in agreement with the low yields of maleimides generally obtained from CrO_3 oxidation of porphyrins (31).

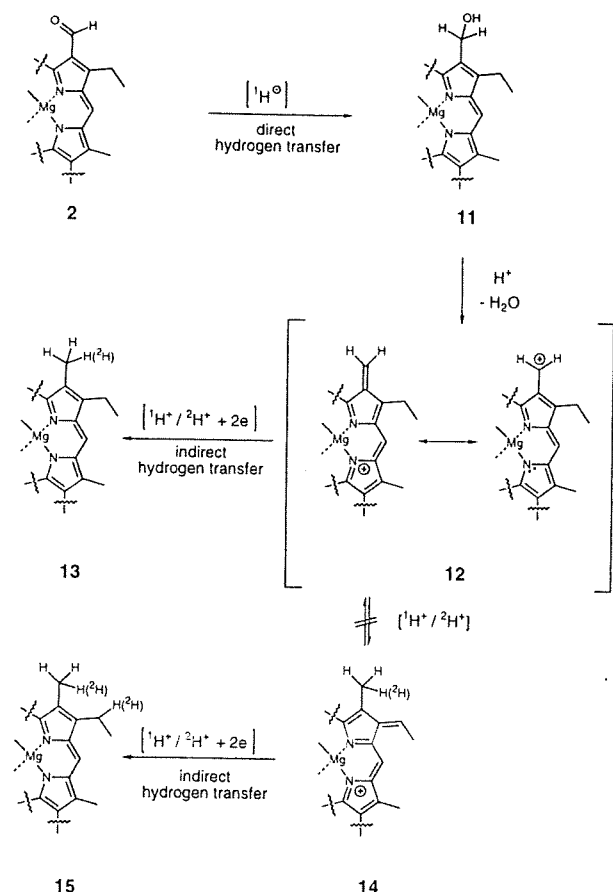


FIG. 8. Proposed two step reduction of Chl *b* to Chl *a* involving a direct and an indirect hydrogen transfer. The reduction of the formyl group of Chl *b* to 7¹-hydroxy Chl *a* (11) is mediated by NAD(P)H. The cofactor is regenerated by hydride transfer from a carbon-bond hydrogen source, which was acquired during the phototrophic growth phase of the plant in ordinary water. Elimination of water from the intermediate 11 affords the resonance-stabilized carbocation 12, which is subsequently reduced by two electrons to the corresponding carbanion. Proton/deuteron quenching of the latter with the isotopic composition of the water yields 13, which then contains the observed mono-deuterated methyl group. Isomerization of the carbocation 12 to 14 and following reduction would introduce label in the ethyl group of 15 that has not been observed. For clarity, only the eastern half of the Chl *b* molecule is shown.

DISCUSSION

The ratio unlabeled to labeled **9** reflects both the isotopic composition of the deuterium concentration in the medium and the exchange and hydrogen transfer reaction, which occur during biodegradation. Formally two hydride ions are required to reduce an aldehyde group to a methyl group. Depending on the mechanism, up to two deuterium atoms per methyl group can be incorporated during the catabolic process of Chl *b* in the presence of heavy water (Fig. 7). We found that only one deuterium atom was incorporated.

This fact demonstrates that of the hydrogen isotopes forming the methyl group of the labeled Chl *a* catabolite one originates from the formyl group of the former Chl *b*, one arrives by hydride transfer from an as yet unidentified carbon-bond hydrogen source, and another enters the methyl group as proton/deuteron from the surrounding water (Fig. 8). Enzymatic reduction of an aldehyde group to the corresponding alcohol is generally accomplished by nicotinamide-dependent hydride carriers NAD(P)H (32, 33). It is a hallmark of these coenzymes not to exchange with the deuterium of the surrounding medium (34). We assume that a hydride ion is mediated by a NAD(P)⁺/

NAD(P)H between a carbon-bond hydrogen such as for example (poly)saccharides, which were acquired during the phototrophic growth phase of the plant in ordinary water and the formyl group. This assumption is supported by *ex vivo* experiments in which NAD(P)H was essential to reduce Zn(II) pheophorbide *b* to the corresponding 7¹-hydroxy-Chl *a* (6).

Reductive elimination of a hydroxyl group is generally a more difficult task due to the strong carbon-oxygen bond. This is the reason why enzymatic examples are rarely found in the literature (5, 35). Nevertheless, the unique electronic arrangement of the cyclic 18- π electron porphyrin system facilitates the formation of a resonance-stabilized carbocation **12** (Fig. 8). This elimination process demands an activator capable to transform the hydroxyl group into a better leaving group. In this context it is noteworthy that ATP, which can act as activator was required for the transformation of chlorophyllide *b* to Chl *a* in barley etioplasts (5) but not for the first reduction step to 7¹-hydroxy Pheo *a* (7). Most recently, it has been demonstrated that the final reduction step is achieved when reduced spinach ferredoxin is added to lysed etioplasts (36). Ferredoxins participate in electron transfer reactions, electrons are typically provided by an electron transfer chain involving NADH and/or flavoproteins (33). Therefore, we regard cation **12** as terminal electron acceptor, which becomes reduced by two electrons to the corresponding carbanion. Final quenching of the latter with a proton/deuteron from the aqueous medium would account for the observed statistically mono-deuterium labeling of the methyl group. It has been suggested that **12** and/or **14** (Fig. 8) are possible intermediates (37). However, ¹H- and ²H-NMR spectra show no deuterium label in the ethyl group of the maleimide fragment **9**; isomerization occurs, if at all, only very slowly.

The catabolic sequence Chl *b* \rightarrow Chl *a* \rightarrow Chl *a* catabolite is more likely to proceed than a subsequent conversion of an assumed Chl *b* catabolite for the following reasons: (i) Pheo *a* oxygenase appears to be highly specific, the enzyme uses Pheo *a* as substrate but refuses Pheo *b* (*vide supra*). (ii) Independent proofs have shown that the Chl *a*(*b*) converting enzymes are present in higher plants (*vide supra*). (iii) The proposed cation **12** is stabilized by resonance through the extended electronic 18 π -system of the Chl macrocycle, whereas the formation of a corresponding cation from 7¹-hydroxy-Chl *b* catabolite would be less favored by resonance.

Chromic acid oxidation of the bile-pigment-like chlorophyll catabolite **5** of *H. vulgare* present in the enriched plant extract afforded in addition to **9** the corresponding maleimides **8** and **10** (Fig. 2). ¹H- and ²H-NMR spectroscopic investigation showed that of the three maleimides isolated from the plant extract only maleimide **9** was partially mono-deuterium labeled at C(4¹), all others (data not shown) were not. During *de novo* biosynthesis of the Chls all methyl groups should become evenly mono-deuterated in heavy water because of the consecutive decarboxylation of uroporphyrinogen III to coproporphyrinogen III by uroporphyrinogen III decarboxylase. This result confirms apart from Chl *a*(*b*) interconversion the general assumption that angiosperms form no Chls in the dark (17).

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Chlorophyll catabolism: High stereoselectivity in the last step of the primary ring cleaving process

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Abstract Green cells of the chlorophyte *Chlorella protothecoides* first cultivated under mixotrophically conditions in a medium containing heavy water (59 atom% ^2H) excrete during the subsequent glucose bleaching process in ordinary water characteristically labeled red chlorophyll catabolites into the culture medium. ^1H -NMR spectroscopic analysis of the methyl ester derivative of the chlorophyll *a* degradation product reveals that the final step of the primary ring opening process of the chlorophylls is highly stereoselective. Moreover, the integrity of the deuterium label on the formyl group which originates from the former C(5) methine group of the chlorophyll macrocycle excludes the formation of a 5-oxophlorin intermediate in the course of the ring opening process.

Key words Chlorophyll, catabolism, degradation, metabolism, *in vivo* deuterium labeling, stereoselectivity, *Chlorella protothecoides*.

Abbreviations BOP, benzotriazol-1-yloxytris(dimethylamino) phosphonium hexafluorophosphate; Et_3N , triethylamine; FID, free induction decay.

INTRODUCTION

The importance of stereochemistry in understanding the mechanism of biological processes has long been recognized (Ogston, 1948). Chiral and/or prochiral centers generated during metabolism are of particular value, because they not only reveal the operating reaction mechanisms but present also sensitive probes for the characterisation of the participating enzymes. The present work provides a first insight in the stereochemical course of the chlorophyll catabolism in a green alga and demonstrates that the last step of the primary ring opening of the chlorophylls (Gossauer and Engel, 1996) is irreversible and highly stereoselective.

The first unequivocally characterized degradation products of chlorophyll *a* had been isolated from a chlorophyte [*Chlorella protothecoides* Krüger] (Engel *et al.*, 1991) and from angiosperms [*Hordeum vulgare* L.] (Kräutler *et al.*, 1991). Later on, catabolites have been found in senescent cotyledons [*Brassica napus* L.] (Mühlecker and Kräutler, 1996) and autumnal leaves of deciduous trees [*Liquidambar styraciflua* L., *L. orientalis* Mill. (Iturraspe *et al.*, 1995) and

Cercidiphyllum japonicum Sieb. et Zucc. (Curty and Engel, 1996)]. Significantly, although chlorophyll catabolites have been obtained from diverse plant divisions (Chlorophyta and Anthophyta) and under different conditions, all catabolites characterized until now are 4,5-dioxo-4,5-secopheophorbide derivatives, *i.e.* linear tetrapyrroles, which result from a regioselective oxidative cleavage at the C(4)-C(5) bond of their cyclic precursor, either chlorophyll *a* or *b*. However, although the constitutional formulae of most of these catabolites have been unequivocally elucidated, the absolute configuration of the asymmetric C-atoms not present in the chlorophylls as well as the stereoselectivity of the enzymatic reactions which introduce prochiral centers in the molecules still remain to be determined. Indeed it is worth mentioning, in this connection, that the shape of the CD spectra of the catabolites isolated from *H. vulgare* (Kräutler *et al.*, 1992) and *C. japonicum* (Curty and Engel, 1996), which belong to different plant classes (monocotyledons and dicotyledons, respectively) is similar, thus suggesting that the absolute configuration of all asymmetric C-atoms present in both compounds is the same.

Fossil record, morphological and physiological characteristics as well as comparison of nucleotide sequences of the 18S and 28S components of cytoplasmatic rRNAs indicate that the higher plant cell has evolved from a chlorophytic ancestor (van den Hoek *et al.*, 1995). Accordingly, the catabolites isolated from today's chlorophyte *C. protothecoides* may have preserved a rudimentary structure when compared with the catabolites isolated from diverse higher plant families. Moreover, the main red chlorophyll *a* degradation product of *C. protothecoides* can be chemically transformed by hydrogenation and subsequent prototropic rearrangement into the skeleton of the catabolites isolated from higher plants (Engel *et al.*, 1996). It is therefore not unlikely that the catabolites isolated from the green alga also occur in higher plants, as primary intermediates. The elucidation of the stereoselectivity of the catabolic process in different divisions and classes is of particular interest, since homochirality in the degradation products would not only consolidate

the phylogenetic relationship between them but also strongly support the evolution of the catabolic pathway for the chlorophylls valid for the whole green plant lineage.

Recently, we evidenced by means of *in vivo* $^{18,18}\text{O}_2$ labeling experiments that a monooxygenase is involved in the catabolism of chlorophyll *a* in the green alga *C. protothecoides*, which most likely catalyzes the formation of an C(4)-C(5) oxirane intermediate (Curty *et al.*, 1995). The final step in the primary cleaving process was suggested to be a prototropic rearrangement in which a proton approaches either spontaneously or enzymically catalyzed one of the diastereotopic faces of the C(10) methine group of the secopheophorbide derivative **4**, thus forming a prochiral center (*fig. 1*). The goal of the present work is to elucidate whether the transformation of the C(10) methine group into a methylene bridge is a stereoselective process or not.

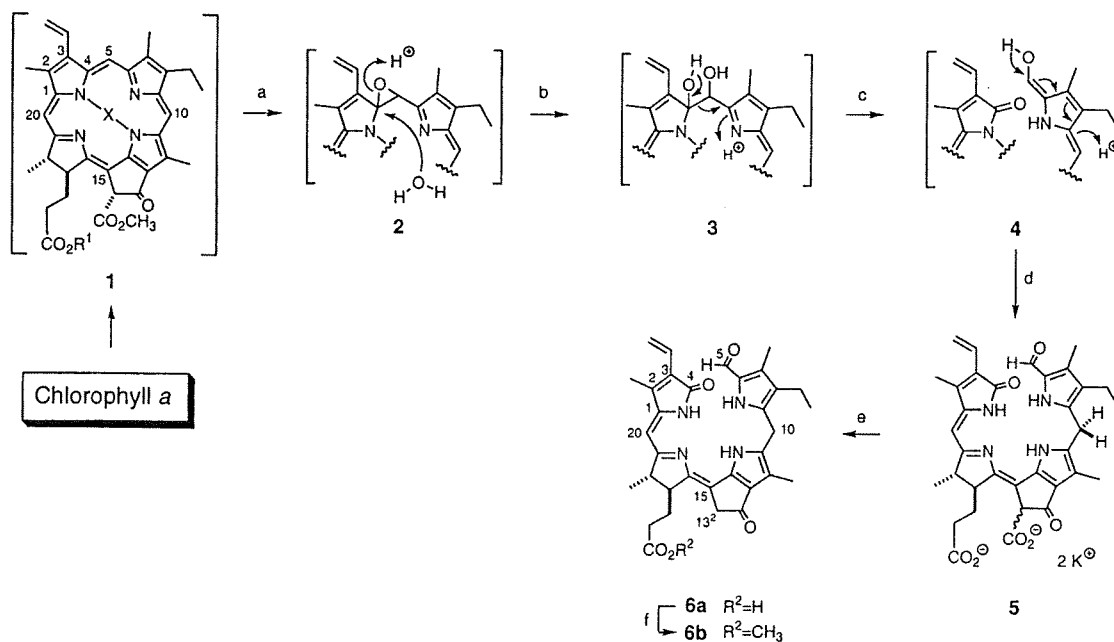


Figure 1. Probable pathway for the primary ring opening process of the chlorophylls (upper part). *a*, regioselective addition of a monooxygenase-activated oxygen molecule to the C(4)-C(5) bond; *b*, regioselective hydrolysis of the formed oxirane derivative; *c*, *retro*-aldol type ring cleavage of the macrocycle; *d*, prototropic rearrangement, in which the proton preferably approaches from only one face of the heterotopic methine group (see text). As the specific substrate of the monooxygenase is still unknown X represents either Mg or 2H and R^1 either phytol or H. The suggested mechanism is based on $^{18,18}\text{O}_2$ labeling experiments, chemical plausibility and analogy to known enzymatic reactions (Engel *et al.*, 1996). For the sake of clarity only the part of the molecule which is modified during the reaction is represented in the partial structures **2-4**. Lower part: reactions which lead to the isolation of the main degradation product of chlorophyll *a* (**6b**): *e*, decarboxylation of the C(13²) carboxylic group during work-up; *f*, esterification of the isolated pigment with MeOH in the presence of BOP and Et_3N (Engel *et al.*, 1996).

RESULTS

In vivo deuterium labeling of *C. protothecoides*

Monoclonal *C. protothecoides* cells were cultured under mixotrophic conditions in water containing 59 atom% ^2H , as determined by ^1H -NMR spectroscopy, and unlabeled D-glucose. During growth the whole algae including the photosynthetic pigments becomes labeled. In the chlorophylls, protium and deuterium are expected to be statistically distributed over all possible positions (*vide infra*). To exchange residual $^2\text{H}_2\text{O}$, the green algal cells were harvested and, thereafter, equilibrated for 3 d in darkness in a medium made with $^1\text{H}_2\text{O}$. During the subsequent culturing in $^1\text{H}_2\text{O}$ under nitrogen starvation in a medium containing unlabeled D-glucose, the alga secrete the red chlorophyll *a* and *b* catabolites into the medium. After 5 d the main chlorophyll *a* degradation product **6a** was isolated and esterified in a system consisting of methanol, BOP and Et_3N , yielding **6b** (Engel *et al.*, 1996). The thus introduced unlabeled methoxy group provided the internal reference for the ^1H -NMR spectroscopic determination of the relative amounts of protium and deuterium present at the labeled positions.

 ^1H -NMR spectroscopic investigation of the chlorophyll *a* catabolite

Integration of the ^1H -NMR spectrum of labeled **6b** reveals: (a) the deuterium label is completely replaced by H-atoms at the C(13²) methylene group and the three nitrogen atoms. These positions are prone to hydrolytic exchange due to their acidity; (b) the intensities of all other proton resonances, except those of the C(10) methylene group (*vide infra*), are diminished to a value close to the expected content of 59 atom% deuterium per position. Remarkably, the label of the original C(5) methine bridge of the macrocycle remains unaffected in the C(5) formyl group of the catabolite; (c) integration over the range between δ 3.9 and 4.0 ppm, in which the protons of the C(10) methylene group of **6b** do absorb, yields an intensity of 1.41 protons. This figure corresponds exactly to the addition of *one* hydrogen to the C(10) methine bridge of the substrate (**4**), which like the other positions is ^2H -labeled to 59% (the original ^2H content of the chlorophyll *a* used for the experiment). This result demonstrates, at once, the stability of the label on this position during the catabolic process as well as during

work up of the culture medium and subsequent esterification.

After resolution enhancement by Gaussian multiplication of the FID (Sanders and Hunter, 1990; Thiele *et al.*, 1994) the signals assigned to the C(10) methylene group of the catabolite (**6b**) were resolved into an apparent ($J_{\text{H-D}} \leq 2.5$ Hz) singlet at δ 3.920 ppm and an AB spin system ($J_{\text{H-H}} = 16.1$ Hz), the two doublets of which are centered at δ 3.957 and 3.938 ppm. The latter originate from the fraction of molecules devoid of label at C(10) (*fig. 2*). As expected from the above

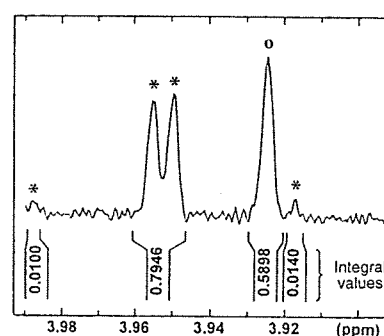


Figure 2. ^1H -NMR (500 MHz in CDCl_3) of partially deuterated **6b** in the range of resonance of the hydrogen atoms bound to C(10). The spectrum was obtained after Gaussian multiplication [Gaussian pseudo echo, LB=0.5, GL=1.0, GB=50] (Sanders and Hunter, 1990; Thiele *et al.*, 1994) of the free induction decay (FID). The fraction of the molecules unlabeled at C(10) give rise to an AB spin system, the components of which are indicated by (*). The apparent singlet (o) originates from the monodeuterated molecules at C(10) in the same sample. The integral values are relative to the signals of the methyl ester group (= 3H).

total intensity of the $\text{H}_2\text{C}(10)$ signals, the integral value of 0.82 for both doublets reveals that 41% of the molecules carry an unlabeled C(10) methylene group (*fig. 3*). Moreover, addition of unlabeled **6b** to the sample gives rise to an increase of the intensity of the four signals of the AB spin system, whereas the intensity of the singlet at δ 3.920 remains unchanged. According to the integral value of this singlet, about 59% of the molecules contain a $^2\text{H}^1\text{HC}(10)$ group. As both hydrogens are diastereotopic, the presence of only one singlet evidences that all molecules containing a $^2\text{H}^1\text{HC}(10)$ group have the same absolute configuration at this chiral center. Actually, evidence against a fortuitous isochronism of the methylene protons in the two possible isotopic C(10) epimers had been obtained earlier from the ^1H -NMR spectrum of mono-

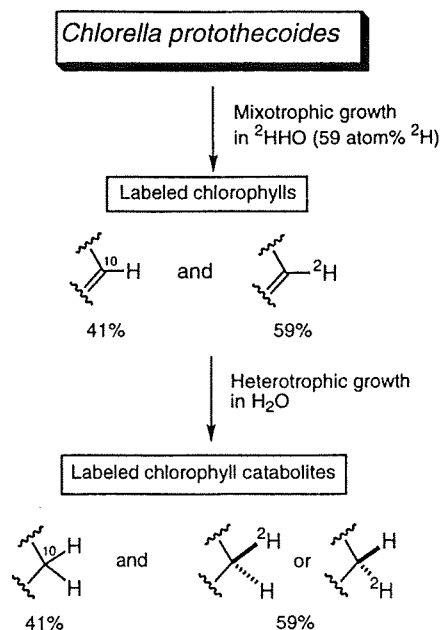


Figure 3. Fate of the deuterium label in the chlorophylls and the catabolites during growth and bleaching of *C. protothecoides*, respectively. For clarity, only the ligands at C(10) are shown.

deuterated **6b**, which was obtained by chemical partial synthesis (Iturraspe and Gossauer, 1992). In this case, two C(10) epimers were obtained in a ratio of 3:2, as evidenced by the presence of two apparent singlets clearly separated from each other by $\Delta\delta$ 0.024 ppm.

Relative to the resonance frequencies of the two protons of the unlabeled C(10) group, the singlet at δ 3.920 ppm is upfield shifted by $\Delta\delta$ 0.037 and 0.018 ppm. Both values are within the range commonly observed for the primary isotope effect due to the difference in magnetic shielding of deuterium and hydrogen attached to the same C-atom (Davis *et al.*, 1988), so that a correspondence between the singlet at δ 3.920 ppm and one of the components of the AB-spin system cannot be established.

DISCUSSION

Uniformly labeled chlorophyll *a* required for the ^1H -NMR spectroscopic analysis of the catabolites obtained after enzymic degradation was acquired by *in vivo* deuterium incorporation of *C. protothecoides* cells in $^2\text{H}_2\text{O}$. The option of labeling the catabolites by

carrying out the degradation process in heavy water, would demand twice as much $^2\text{H}_2\text{O}$. Although highly deuterated chlorophylls and other metabolites have been previously obtained from *Scenedesmus obliquus* and *Chlorella vulgaris* when grown in heavy water containing up to 99 atom% ^2H (Thomas, 1971; Mohan and Mahadevan, 1993) the experiments with *C. protothecoides* were performed at a lower $^2\text{H}_2\text{O}$ concentration in order to maintain reasonable growth rates. Actually, in 99 atom% heavy water, this alga failed to grow at all.

In the present study we demonstrated by ^1H -NMR spectroscopic analysis of the deuterium labeled chlorophyll *a* catabolite isolated from *C. protothecoides*, that all molecules containing a $^2\text{H}^1\text{HC}(10)$ group have the same absolute configuration. Albeit the absolute configuration at C(10) of the labeled catabolite **6b** remains unknown, the results prove that the final step in the primary cleaving process is completely stereoselective and, therefore, most likely enzymically controlled. For the conclusion drawn from this investigation it is irrelevant whether a proton (deuteron), as suggested, or another H-species (*i.e.* a hydrogen atom or a hydride ion) is finally added to the C(10) methine group of the molecule. It should further be noted, that the integrity of the label on the formyl group which originates from the C(5) methine group of the chlorophyll macrocycle excludes the formation of a 5-oxophlorin intermediate, as it is supposed to occur in heme catabolism (Beale, 1993). This supports our previously suggested mechanism of oxidative ring cleavage as the primary step in the catabolic process of chlorophylls (Curty *et al.*, 1995).

METHODS

Plant material. The Chlorophyte *Chlorella protothecoides* Krüger denoted ACC #25 is maintained in our laboratories on a standard agar slant and was the same as previously described.

***In vivo* labeling of *C. protothecoides*.** All manipulations were performed aseptically, centrifugations were at 2000 x g and all salt-solutions were prepared with ordinary deionized water ($^1\text{H}_2\text{O}$). The green alga culture medium consisted of: $^2\text{H}_2\text{O}$ (75 ml, 80 atom% ^2H , Armar, Switzerland), H_2O (25 ml), KH_2PO_4 (680 mg), K_2HPO_4 (871 mg), $(\text{NH}_4)_2\text{HPO}_4$ (1.5 ml, 1 M), FeSO_4 (0.1 ml, 0.01 M), MgSO_4 (0.1 ml, 1.2 M), oligoelement solution Arnon A5 (0.1 ml), CaCl_2 (0.1 ml, 0.1 M), vitamin solution (Nitsch and Nitsch, Sigma, product no. N 8764) (0.1 ml) and unlabeled D-glucose (0.1 ml).

cose x $^1\text{H}_2\text{O}$ (0.5 g). The final aqueous medium contains 59 atom% ^2H , determined by ^1H -NMR. The resulting solution was sterilized by membrane filtration (0.2 μm , Schleicher and Schüll no. 00040) and incubated with monoclonal axenic cells of the green alga *C. protothecoides*. The alga was shaken on an orbital shaker at 135 rpm for 13 d at 25°C under continuous illumination with fluorescent lamps (Sylvania GTE, Germany) at about 4000 lux. To increase the cell number a sterile filtered solution of D-glucose x $^1\text{H}_2\text{O}$ (0.5 g), oligoelement solution Arnon A5 (0.1 ml) in $^2\text{H}_2\text{O}$ / $^1\text{H}_2\text{O}$ (4 ml, 59/41) was added and incubated for additional 2 d, maintaining the same conditions as above.

Glucose bleaching and isolation of the main chlorophyll degradation product. After centrifugation the green cells were equilibrated in 100 ml potassium phosphate buffer (0.1 M, pH 6.8) under shaking for 3 d at 25°C in darkness to exchange residual $^2\text{H}_2\text{O}$. The cells after centrifugation were washed with potassium phosphate buffer (100 ml, 0.1 M, pH 6.8). The centrifuged cells were incubated in a medium containing in 200 ml H_2O , KH_2PO_4 (1.36 g), K_2HPO_4 (1.74 g), FeSO_4 (0.2 ml, 0.01 M), MgSO_4 (0.2 ml, 1.2 M), oligoelement solution Arnon A5 (0.2 ml), CaCl_2 (0.2 ml, 0.1 M), vitamin solution (Nitsch and Nitsch) (0.2 ml), and D-glucose x $^1\text{H}_2\text{O}$ (10 g) and shaken in darkness at 135 rpm for a period of 5 d at 25°C. The bleached cells were removed by centrifugation at 4000 x g. Isolation of the main red chlorophyll *a* degradation product from the supernatant, esterification with methanol and purification by thick-layer chromatography was performed as previously described (Engel *et al.*, 1991; Engel *et al.*, 1996).

Instrumentation. ^1H -NMR measurements were performed in CDCl_3 with a Bruker Avance DRX-500 instrument. Chemical shifts δ are given in ppm downfield from tetramethylsilane, as internal standard. For measurements a resolution of 4.89×10^{-4} ppm per data point has been selected.

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Phytoplankton pigments in oceanography: guidelines to modern methods

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3

Metabolism and function of photosynthetic pigments

R. J. Porra, E. E. Pfündel and N. Engel

3.1 INTRODUCTION

3.2 PIGMENT FUNCTION IN PHOTOSYNTHESIS

- 3.2.1 *Pigment-protein complexes*
- 3.2.2 *Assembly of pigment-protein complexes into LHCs and RCs in thylakoids*
- 3.2.3 *Function of pigment-protein complexes in Photosystems I and II*

3.3 ROLE OF CHLOROPHYLLS IN PHOTOSYNTHETIC LIGHT TRANSDUCTION TO CHEMICAL ENERGY

- 3.3.1 *Light-harvesting and energy transfer to Reaction Centres*
- 3.3.2 *Charge separation and photosynthetic electron transport*
- 3.3.3 *Molecular composition of pigment-protein complexes*

3.4 NATURALLY OCCURRING CHLOROPHYLLS

3.5 BIOSYNTHESIS OF CHLOROPHYLLS AND PHYCOBILINS

- 3.5.1 *Significance and mechanisms of 5-aminolevulinate formation*
 - 3.5.1.1 *ALA-synthase pathway*
 - 3.5.1.2 *C₅-pathway of ALA formation*
- 3.5.2 *Conversion of ALA to protoporphyrin IX*
- 3.5.3 *Protoporphyrin IX: bifurcation point for iron and magnesium branches of tetrapyrrole formation*
- 3.5.4 *Iron branch: formation of phycobilins via protohaem IX*
 - 3.5.4.1 *Formation of protohaem IX by ferrochelatase*
 - 3.5.4.2 *Formation of phycobilins*
- 3.5.5 *Magnesium branch: conversion of protoporphyrin IX to chlorophylls a and b*
- 3.5.6 *Formation of chlorophyll c pigments*
- 3.5.7 *Chlorophyll and carotenoid incorporation into photosynthetic pigment-protein complexes*

3.6 CHEMICALLY ALTERED CHLOROPHYLLS

- 3.6.1 *Allomerization of chlorophylls*
- 3.6.2 *Epimerization at C-13²*
- 3.6.3 *Demetallation reactions*
- 3.6.4 *Dephytylation*
- 3.6.5 *Chlorination of chlorophylls*
- 3.6.6 *Decarboxymethylation at C-13²*

3.7 BIODEGRADATION OF CHLOROPHYLLS

- 3.7.1 *Cyclic tetrapyrrolic catabolites*
- 3.7.2 *Linear tetrapyrrolic catabolites*
 - 3.7.2.1 *Catabolites from higher plants*

Phytoplankton pigments in oceanography: guidelines to modern methods

- 3.7.2.2 *Catabolites from freshwater green algae*
 - 3.7.2.3 *Unusual chlorophyll catabolites from marine organisms*
 - 3.7.3 *Methods of identification of linear catabolites*
 - 3.7.3.1 *UV/VIS spectroscopic properties*
 - 3.7.3.2 *Chromic acid test*
- 3.8 BIOSYNTHESIS AND FUNCTION OF CAROTENOIDS
 - 3.8.1 *Biosynthesis of carotenoids*
 - 3.8.1.1 *Intracellular distribution of enzymes of carotenoid biosynthesis*
 - 3.8.1.2 *Early enzymes of carotenoid biosynthesis and the formation of isopentenyl diphosphate*
 - 3.8.1.3 *Later enzymes converting IPP to carotenes*
 - 3.8.1.4 *Formation of xanthophylls*
 - 3.8.2 *Regulation of carotenoid biosynthesis*
 - 3.8.3 *Biological degradation of carotenoids*
 - 3.8.3.1 *Senescence processes*
 - 3.8.3.2 *Examples of chemical modification of carotenoids in animals*
 - 3.8.4 *Function of carotenoids*
 - 3.8.4.1 *Free carotenoids and protein complexes binding carotenoids exclusively*
 - 3.8.4.2 *Carotenoids in chlorophyll pigment-protein complexes*
 - 3.8.4.3 *Light-harvesting properties*
 - 3.8.4.4 *Photoprotection by carotenoids*
 - 3.8.4.5 *Protection against photoinhibition by xanthophyll cycles*
- 3.9 SUMMARY

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REFERENCES

ABBREVIATIONS

ABA, abscisic acid; ALA, 5-aminolevulinic acid; ATP, adenosine triphosphate; BChl, bacteriochlorophyll; BPhe, bacteriopheophytin; Chl, chlorophyll; Chlide, chlorophyllide; CP, proteins of PSI or PSII core complexes; DMAPP, dimethyl allyl diphosphate; GGPP, geranylgeranyl diphosphate; GSA, glutamate-1-semialdehyde; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; IPP, isopentenyl pyrophosphate; LHC I and II, light-harvesting complexes of photosystems I and II; LHCP, light-harvesting Chl *a/b* proteins; NAD⁺, nicotine adenine dinucleotide; NADH, reduced nicotine adenine dinucleotide; NADP⁺, nicotine adenine dinucleotide phosphate; NADPH, reduced nicotine adenine dinucleotide phosphate; PAR, photosynthetically active radiation; PBG, porphobilinogen; PChlide, protochlorophyllide; PS I and PS II, photosystems I and II; RC, reaction centre; TCA, tricarboxylic acid cycle

The tetrapyrrole nomenclature and macrocycle numbering system (Moss, 1987) approved by IUPAC-IUB is used throughout this chapter.

3.1 INTRODUCTION

This chapter provides an introduction to the properties, biosyntheses and functions of the major pigments of photosynthesis. It also discusses the nature, origin and function of the many carotenoid and tetrapyrrolic chromophores and their derivatives, and the degradation products that may be encountered in phytoplankton extracts pre-

3.6.6 Decarboxymethylation at C-13²

The C-13²-carboxymethyl group can be removed by heating anaerobically in pyridine at 100°C for 24 h to form pyrochlorophylls. Decarboxymethylation can also occur enzymically (see section 3.7).

3.7 BIODEGRADATION OF CHLOROPHYLLS

Global Chl degradation is about 10⁹ tonnes annually of which about 75% occurs in oceans, lakes and rivers (see section 3.2). Chl derivatives are formed by grazing and digestion by terrestrial and aquatic herbivores, and also by cell senescence and microbial degradation. Until recently, the disappearance of so much Chl without the formation of detectable intermediates or end products was regarded as an enigma (Hendry *et al.* 1987). Useful reviews of Chl biodegradation include those of Simpson *et al.* (1976), Kufner (1980), Rüdiger and Schoch (1989) and Brown *et al.* (1991).

The products of biological degradation are classified here into two categories:

- peripherally modified cyclic tetrapyrroles (see section 3.7.1), and
- linear tetrapyrroles from oxidative cleavage of Chl macrocycles (see section 3.7.2).

3.7.1 Cyclic tetrapyrrolic catabolites

In many Chl degradation products, the tetrapyrrole macrocycle remains intact. Enzymes implicated in such degradative processes catalyze pheophytinization, phytyl ester hydrolysis, decarboxymethylation and allomerization. In contrast to the peripheral substituents, the macrocycle is remarkably stable, particularly when oxygen and light are excluded. Some metal complexes of phorbins (five-ring macrocycle; see section 3.4) have survived chemical modification over a long geochemical time scale (diagenesis), losing only the phytyl ester and thus surviving as 'molecular fossils' in a wide range of sediments dating back to the Precambrian period (Callot, 1991). This field of petroporphyrin geochemistry, based on the work of Treibs in the early 1930s, has exploded recently with the availability of sensitive analytical methods for separation and structure determination of petroporphyrins and petrochlorins. While some of these compounds (over 70 are now known; Callot, 1991) can be related to known Chl precursors (e.g., Chls *b*, *c*), others remain an enigma, possibly indicating unknown Chl precursors still awaiting discovery in present-day aquatic ecosystems.

Chlorophyllase, which readily hydrolyses the phytyl ester bond of Chls under physiological conditions, was first discovered by Willstätter and Stoll (1910), and it has been detected in higher plants (Mayer 1930) and phytoplankton (Barrett and Jeffrey, 1964; Jeffrey and Hallegraeff, 1987).

Magnesium dechelataase catalyzes the removal of the magnesium ion (pheophytinization) from all Chls (or Chlides) at neutral pH (Owens and Falkowski, 1982; Ziegler *et al.* 1988).

Pyropheophytins and pyropheophorbides, which arise by removal of the 13²-methylcarboxylate group on *isocyclic* ring E, have been found in photosynthetic organisms (Ziegler *et al.* 1988; Schoch *et al.* 1981), in marine sediments (Keely and Maxwell, 1991; Sun *et al.* 1993), and during grazing (Hawkins *et al.* 1986).

Phytoporphylin (cf Fig. 3.4A) has been isolated from the intestines and faeces of some domestic herbivores (Fischer and Stern, 1943), which indicates that, during

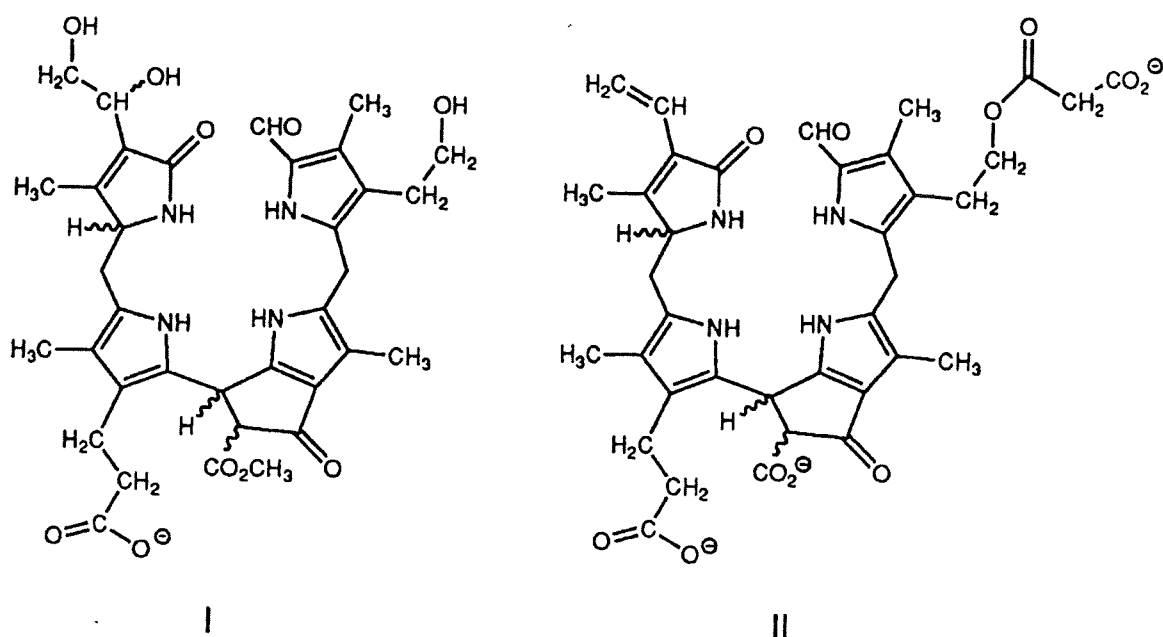


Figure 3.12

Two colourless Chl catabolites, cleaved at C-5, isolated from higher plants I, from *Hordeum vulgare*; II, from *Brassica napus*.

digestion, Chl *a* has been subject to demetallation, dephytylation, reduction of the 3-vinyl substituent, decarboxymethylation and oxidation of ring D. Similar processes occur in marine herbivores (Hawkins *et al.* 1986).

Chl oxidase transforms Chls to their 13²-hydroxy epimers (Schoch *et al.* 1984).

3.7.2 Linear tetrapyrrolic catabolites

Biodegradation of Chls to linear tetrapyrroles in algae and higher plants proceeds mostly by oxidative cleavage of the Chl macrocycle between C-4 and C-5 with the C-5 bridge carbon retained as an aldehyde function. This contrasts with the loss of C-5 as carbon monoxide during phycobilin formation from protohaem IX (see section 3.5.4.2 and compare Figs. 3.8 and 3.12).

Linear tetrapyrroles derived from Chls have been found in higher plants, freshwater chlorophytes and marine organisms. These linear tetrapyrrole catabolites of Chls retain the fifth *isocyclic* ring. They were the first linear tetrapyrrolic catabolites of Chl to be characterized. No linear pyrrole fragments smaller than a tetrapyrrole have been detected.

3.7.2.1 Catabolites from higher plants

Two linear tetrapyrroles (I) and (II) (Fig. 3.12) derived from the Chl *a* of higher plants have been identified by Kräutler *et al.* (1991) and Mühlecker *et al.* (1993), respectively. In both catabolites, the oxidative cleavage of the Chl macrocycle occurred between C-4 and C-5, with retention of C-5 as a formyl function on ring B.

These colourless, water-soluble catabolites have a UV absorption maximum at 320 nm which has been assigned to the formyl-pyrrole moiety. Exposure to air and light during TLC chromatography converts the colourless catabolites to rust-coloured derivatives.

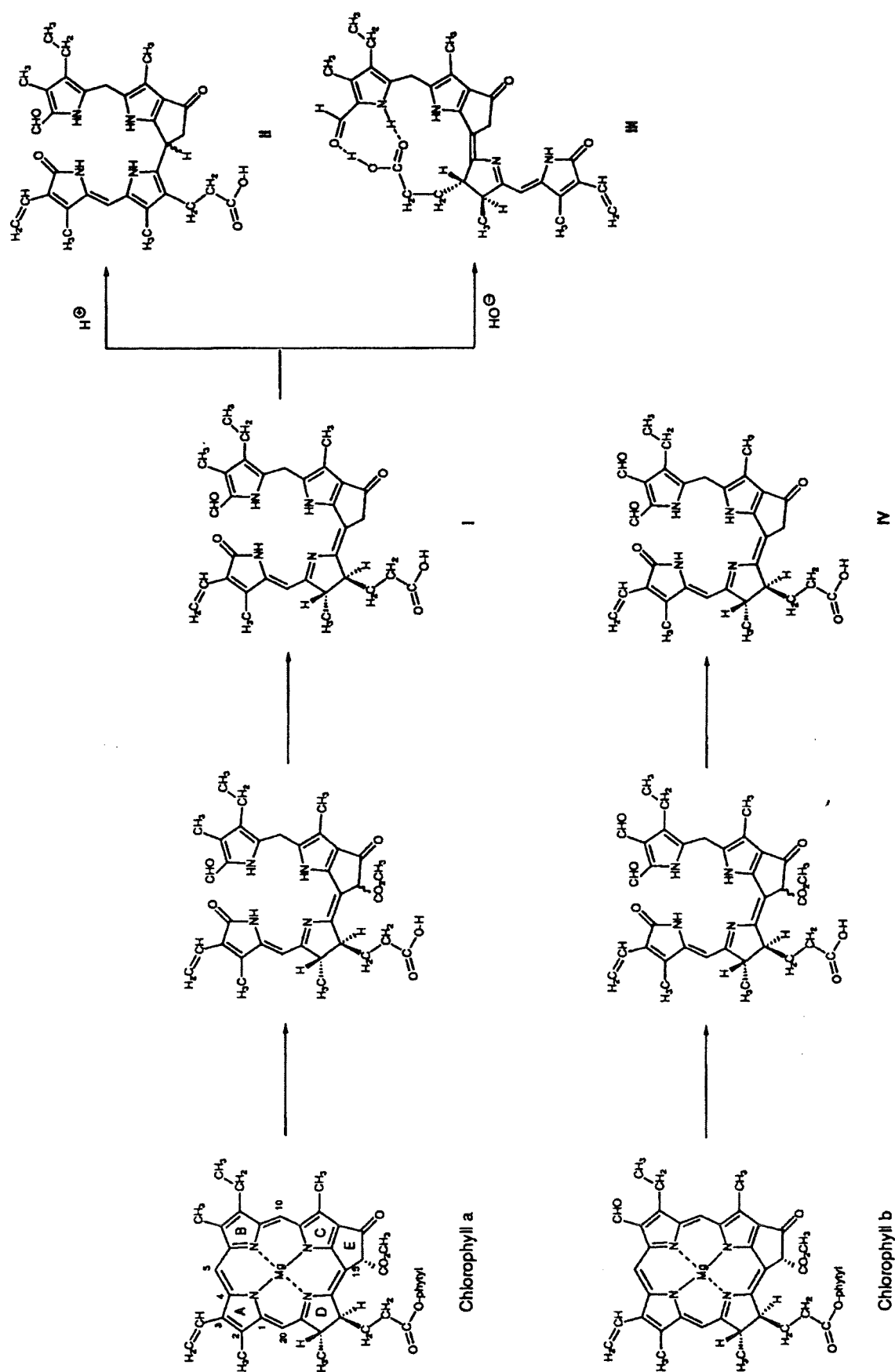


Figure 3.13

A tentative pathway for the degradation of Chls *a* and *b* suggested by the linear tetrapyrrolic catabolites, cleaved at C-5, and isolated from the freshwater alga, *Chlorella protothecoides*. **I** is converted to **II** and **III** by acid and alkaline treatment, respectively, and probably do not represent any enzymic reactions.

3.7.2.2 *Catabolites from freshwater green algae*

The microscopic green alga *Chlorella protothecoides* when grown in media rich in glucose but poor in nitrogen, may excrete several red pigments into the culture medium. These linear tetrapyrrole derivatives of Chls *a* and *b* (Fig. 3.13) are formed by oxidative cleavage between C-4 and C-5, with the 5-formyl group retained on ring B (Engel *et al.* 1991; Iturraspe *et al.* 1993, 1994) as observed in the higher-plant catabolites (see section 3.7.2.1). The red pigment (I) from Chl *a* is unstable: it isomerizes under acidic or basic conditions, leading to either yellow (II) or red (III) pigments, respectively. Being free carboxylic acids, these linear tetrapyrroles are readily soluble in aqueous alkali from which they may be re-extracted into suitable organic solvents by acidification. The purified pigments are readily soluble in alcohols but not in chloroform (Engel *et al.* 1991). Pigment IV is the analogous pigment to pigment I but derived from Chl *b*.

The spectra I, II and III (Fig. 3.14) are the spectra of the methyl esters of compounds I, II and III shown in Fig. 3.13 and discussed in section 3.7.3.1.

3.7.2.3 *Unusual Chl catabolites from marine organisms*

Two linear Chl catabolites (Fig. 3.15) — dinoflagellate luciferin (I) and krill luciferin (II) — are unusual in that they arise from cleavage of Chls at C-20 (Nakamura *et al.* 1988, 1989). They are bioluminescent Chl catabolites found in *Pyrocystis lunula* (dinoflagellates) and *Euphausia pacifica* (krill). As *Euphausia pacifica* is a heterotroph, the linear tetrapyrroles found in these animals must arise from the Chl content of the algal prey.

3.7.3 *Methods of identification of linear catabolites*

NMR-spectroscopy, mass spectrometry and X-ray structure determination have been widely used to determine chlorophyll catabolite structure (Engel *et al.* 1993). UV/VIS spectroscopy and a simple chromic acid test (Rüdiger, 1968, 1969) may also show if the compound under investigation is a tetrapyrrole.

3.7.3.1 *UV/VIS spectroscopic properties*

Native and peripherally altered Chls with an intact macrocycle display the characteristic strong Soret band at about 400 nm. Soret intensity is weaker in the phytochlorins and metallo-phytochlorins than in the porphyrins. All metal-free cyclic tetrapyrroles (other than porphyrinogens) and their Mg-complexes exhibit strong red fluorescence under UV light (see Part IV Data Sheets).

The position of the absorption maxima of linear *oligopyrroles* (Fig. 3.14) is influenced by the 'length' of the conjugated π -electron system (Falk, 1989). Linear tetrapyrroles with two conjugated pyrrole rings (Fig. 3.13 II) display just one main absorption band at about 440 nm and appear yellow (Fig. 3.14; spectrum II). Linear tetrapyrroles with three conjugated pyrrole rings (Fig. 3.13 I and III) bring about a 100 nm shift of the electronic absorption bands to longer wavelengths (about 540 nm) and a short wavelength transition becomes visible (Fig. 3.14; spectra I and III); therefore, solutions of pigments I and III appear reddish. Possession of four conjugated pyrrole rings would cause the long wavelength absorption band to be shifted bathochromically by 100 nm to 600-650 nm and such pigment solutions would appear blueish (e.g., phycocyanin).

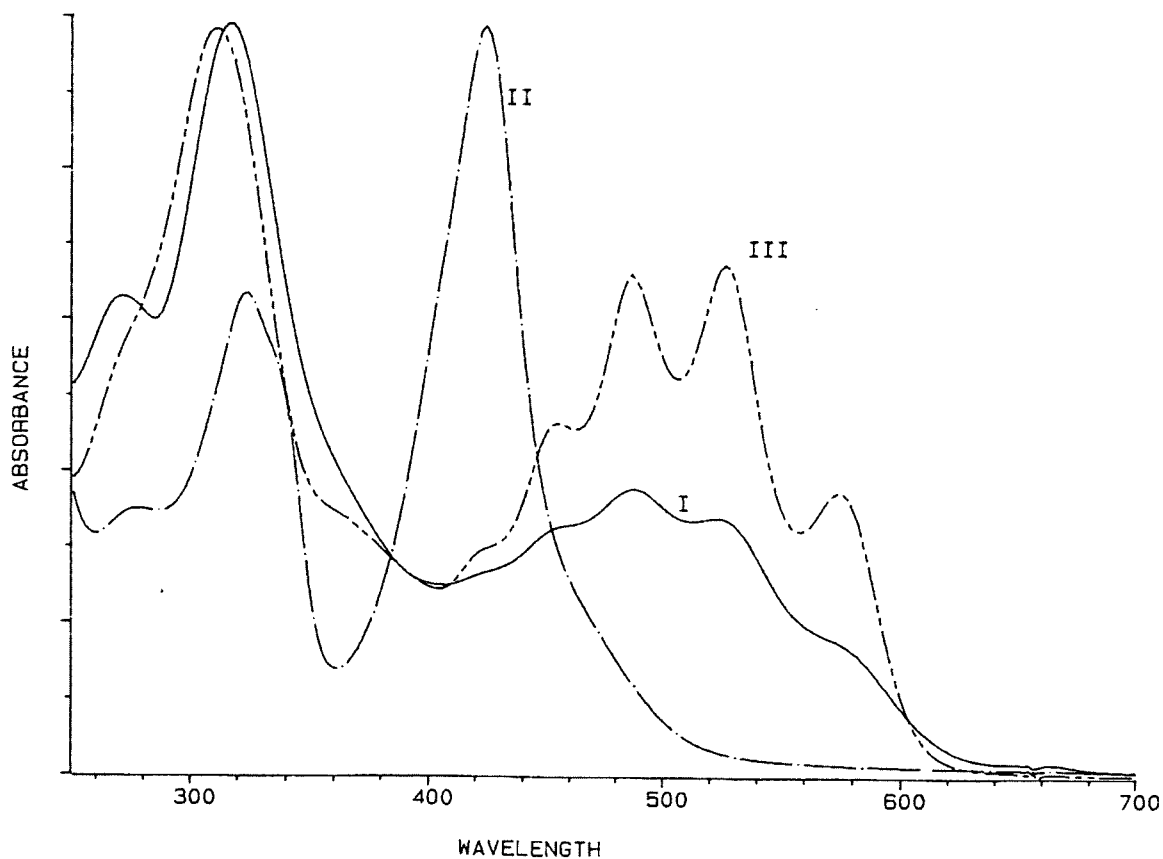


Figure 3.14
Absorption spectra of the methyl esters of algal pigments I, II and III, as designated in Fig 3.13, dissolved in chloroform.

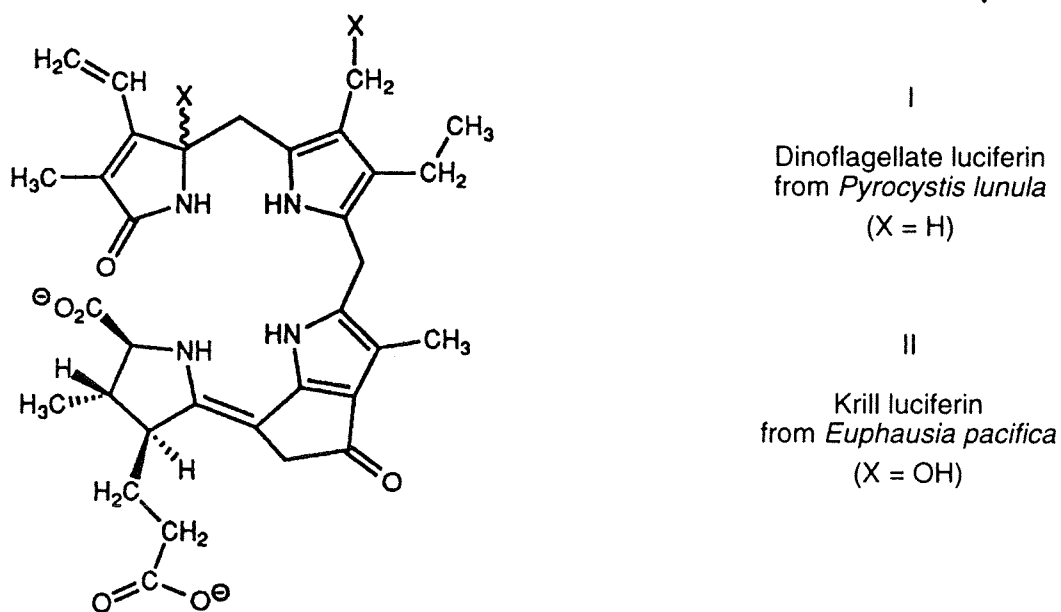


Figure 3.15
Two unusual linear tetrapyrrole catabolites of Chl, cleaved at C-20, and isolated from the marine organisms *Pyrocystis lunula* (dinoflagellate; I) and *Euphausia pacifica* (krill; II).

3.7.3.2 *Chromic acid test*

Besides the non-destructive spectroscopic methods, the chromic acid oxidation test is the method of choice to distinguish Chl- and porphyrin-derived pigments from other chromophores, including anthocyanins and carotenoids. Even non-coloured tetrapyrroles can be made visible by this fast, simple and convenient procedure. Trace amounts ($>0.5 \mu\text{g}$) of these pigments can be detected on TLC-plates by applying a mixture of chromic and sulphuric acids to the origin spot containing the unknown pigments (Rüdiger, 1968, 1969). After the plate is developed in an appropriate solvent, exposed to a chlorine atmosphere and sprayed with benzidine, the oxidation products are visible as blue spots on a white background. By comparing the pyrrolic fragmentation pattern obtained with suitable standards after applying the chromic acid test, it is sometimes possible to deduce the original structure.

3.8 BIOSYNTHESIS AND FUNCTION OF CAROTENOIDS

De novo synthesis of carotenoids is confined to prokaryotes, fungi, algae and higher plants. Approximately 600 different carotenoids are known. Those found in animals are derived from ingested pigments, often chemically modified after ingestion (Thommen, 1971; Goodwin, 1992; Pfander, 1992; Hendry, 1993).

This section briefly introduces the biosynthetic pathway for C_{40} -carotenoids and their function in photosynthesis. Structural relationships between marine algal carotenoids and visible light spectroscopy of carotenoids are discussed in Appendix C and Appendix D. Young and Britton (1993) review the role of carotenoids in photosynthesis.

3.8.1 *Biosynthesis of carotenoids*

In higher plants, mapping of carotenoid biosynthesis genes confirmed their location in the nucleus (Bartley *et al.* 1994). In bacteria, carotenogenic genes are tightly clustered (Sandmann, 1991) in the DNA nucleoid.

3.8.1.1 *Intracellular distribution of enzymes of carotenoid biosynthesis*

Two models for the intracellular distribution of the enzymes of isoprenoid biosynthesis have been suggested (for review see Gray, 1987). The first assumes that chloroplasts are competent to carry out the entire reaction sequence from CO_2 fixation to C_{40} -carotenoids. The second proposes that isopentenyl diphosphate (IPP) is formed in the cytoplasm and then transported to the chloroplast to serve as a carotenoid precursor. As discussed by Gray (1987), experimental evidence for both types of distribution exist.

The inner envelope membrane of the chloroplast was long assumed to be the site of conversion of IPP to carotenoids (Lütke-Brinkhaus *et al.* 1982) but recent work has shown that most phytoene desaturase activity is associated with the thylakoid membranes (Linden *et al.* 1993). The epoxidation of zeaxanthin to violaxanthin was also demonstrated in osmotically-shocked thylakoid preparations, which are envelope-free (Siefermann and Yamamoto, 1975a; 1975b).

Little is known about the distribution of most of the enzymes catalyzing the biosynthesis of C_{40} -carotenoids, because they are integrated within the membrane and difficult to isolate and characterize (Britton, 1990; Sandmann, 1991). They oc-



DETECTION, ISOLATION AND STRUCTURE ELUCIDATION OF A CHLOROPHYLL *a* CATABOLITE FROM AUTUMNAL SENESCENT LEAVES OF *CERCIDIPHYLLUM JAPONICUM**†

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Key Word Index—*Cercidiphyllum japonicum*; Cercidiphyllaceae; chlorophyll *a* catabolism; chlorophyll *a* degradation; autumnal senescence; fall foliage; 19-formyl-1[21H,22H]bilinone.

Abstract—Extracts of autumnal leaves of the dicotyledonous, deciduous tree *Cercidiphyllum japonicum* cultivated at the Botanical Garden of Fribourg, Switzerland, were screened for chlorophyll catabolites by TLC utilizing the chromic acid degradation test. The constitution of the isolated material was elucidated by spectroscopy. The structure, an optically active bile-pigment-like 19-formyl-1[21H,22H]bilinone derivative, reveals that this compound originates from chlorophyll *a* and resembles the structures of previously isolated chlorophyll catabolites from the green alga *Chlorella protothecoides* and from the angiosperms, the monocot *Hordeum vulgare* and the dicot *Brassica napus*.

INTRODUCTION

During autumnal senescence, chlorophyll degradation occurs most impressively in the leaves of deciduous trees and shrubs. The disappearance of the chlorophylls is accompanied by the development of brilliant reds, yellows and golds. The apparent loss of this important chloroplast pigment marks an age-related deterioration of the photosynthetic capacity [3]. Progress has been achieved during the last five years in the elucidation of the molecular structures and the mechanisms involved in the degradation of the chlorophylls. The metabolites which proved to be linear tetrapyrroles were isolated from a chlorophyte *Chlorella protothecoides* Krüger [1], and from angiosperms of the families Gramineae (*Hordeum vulgare* L.) [4, 5], and Cruciferae (*Brassica napus* L.) [6, 7] (Fig. 1).

The green alga *C. protothecoides*, when grown heterotrophically under nitrogen starvation, excretes red chlorophyll *a* and *b* catabolites into the medium [1, 8, 9]. In contrast, excised primary leaves of barley (*H. vulgare*) aged artificially in darkness and cotyledons of the dicot rape (*B. napus*) cultivated under natural photoperiod, accumulate colourless tetrapyrrolic compounds in the vacuoles of the mesophyll cells; only catabolites of chlorophyll *a* have been found to date

[10]. It was surprising to find that in all characterized catabolites regioselective oxidative ring scission occurred at the C(4)-C(5) *meso*-position with retention of C(5) as a formyl group on ring B, yielding 19-formyl-1[21H,22H] bilinone derivatives. *In vivo* labelling experiments performed with *C. protothecoides* cells unequivocally showed that a monooxygenase is involved in the primary cleaving step [11]. Most recently, we proved that the main red pigment 2c isolated from the culture medium of the green alga can be transformed chemically in acidic medium, into a compound showing the same basic skeleton as the colourless breakdown products isolated from both barley and rape. Based on these studies, a generalized mechanism of enzymatic chlorophyll breakdown has been suggested [1, 2], linking the catabolite(s) isolated from the green alga to the catabolites found in higher plants.

We now report the detection, isolation and characterization of a new colourless chlorophyll *a* catabolite from autumnal leaves of the deciduous tree *Cercidiphyllum japonicum* which grows under natural conditions at the Botanical Garden of Fribourg, Switzerland.

RESULTS AND DISCUSSION

The constitution of the catabolite 1 isolated from *C. japonicum* was deduced from ¹H and ¹³C NMR measurements and from mass spectra (see Experimental). FAB-mass spectrometry established molecular ions at *m/z*: 645, 667 (base peak) and at 689, suggesting a

*Part 9 in the series 'Chlorophyll Catabolism'. For Part 8 see [1]. For a recent review see [2].

†Dedicated to Professor Horst Senger on the occasion of his 65th birthday.

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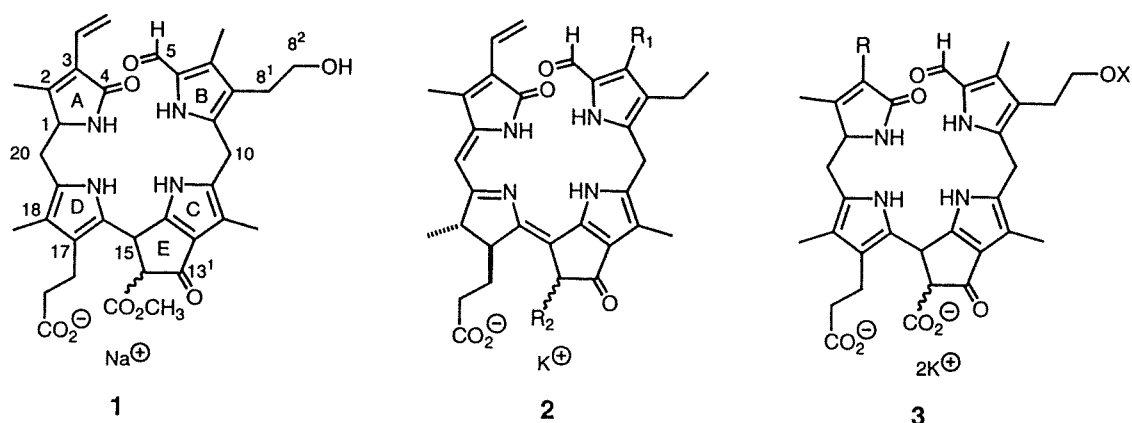


Fig. 1. Catabolites hitherto isolated from: (i) *Cercidiphyllum japonicum* Sieb. et Zucc. **1** [this work]; (ii) the unicellular green alga *Chlorella protothecoides* Krüger; **2a** $R_1 = \text{CH}_3$, $R_2 = \text{COOK}$; **2b**: $R_1 = \text{CHO}$, $R_2 = \text{COOK}$ (both characterized as dimethyl ester) [1, 8]; **2c**: $R_1 = \text{CH}_3$, $R_2 = \text{H}$ (characterized as free acid and as methyl ester) [9]; (iii) barley (*Hordeum vulgare* L.); **3a**: $X = \text{H}$, $R = 1,2\text{-dihydroxyethyl}$, as $O(13^4)\text{-methyl ester}$ [4, 5]; (iv) rape (*Brassica napus* L.), **3b**: $X = \text{H}$, $R = \text{vinyl}$ [7]; **3c**: $X = \text{malonyl}$, $R = \text{vinyl}$ [6]; **3d**: $X = 1\text{-glucosidyl}$, $R = \text{vinyl}$ [7].

tetrapyrrole moiety. Mixing of the sample with KI resulted in additional clusters, centred at m/z of 683 and 721, owing to ion exchange. Quantitative determination of sodium by flame emission spectrometry of the sample dissolved in MeOH showed the presence of equivalent amounts of sodium in the sample, which suggests the existence of a carboxylate group.

In the ^1H NMR spectrum of **1** dissolved in per-deuteriomethanol, 33 of the 39 protons of the compound are seen as resonance signals, of which 19 protons account for one formyl, one vinyl, four methyls and one methoxycarbonyl group (Fig. 2). Connectivities in space are derived from $^1\text{H}\{^1\text{H}\}$ NOE difference experiments (Table 1; Fig. 2). Four amino, one hydroxyl and

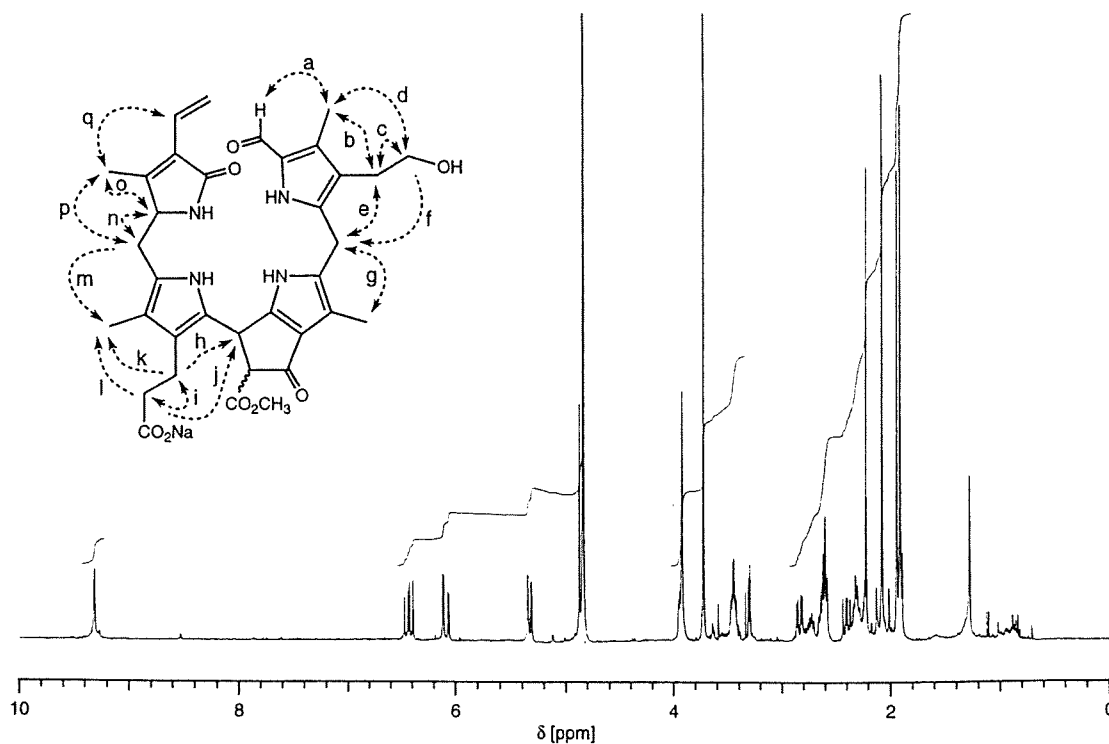


Fig. 2. 360 MHz ^1H NMR spectrum of **1** in CD_3OD showing the purity of the preparation. Owing to the acidity of the proton on C(13²) rapid H/D exchange occurs by the solvent. Therefore, the proton on C(15) at $\delta = 4.88$ ppm appears as a singlet (see text). In the structure the connectivities obtained from homonuclear NOE difference experiments are indicated by arrows (see Table 1).

Table 1. ^1H NMR signals of **1** assigned by $^1\text{H}\{^1\text{H}\}$ NOE difference experiments measured in CD_3OD ($c\ 3 \times 10^{-2}$ M)

Irradiated signals	Enhanced signal †	% Enhancement	Assignments in formula 1 (Fig. 2)
9.31	2.22 (3H, s, H-7 1)	5.0	a
6.43	5.33 (1H, dd, $J = 11.6$, $J = 2.4$, $H_{\text{cis}}-3^2$)	8.7	Not shown
	1.94 (3H, s, H-2 1)	3.5	q
3.94*	2.84 (1H, dd, $J = 14.2$, $J = 4.9$, $H_{\text{B}}-20$)	1.2	n
	2.41 (1H, dd, $J = 14.4$, $J = 9.5$, $H_{\text{A}}-20$)	2.8	n
	1.94 (3H, s, H-2 1)	1.6	o
3.92*	2.68-2.55 (2H, m, H-8 1)	1.7	e
	2.08 (3H, s, H-12 1)	2.0	g
3.44	3.92 (2H, s, H-10)	Small	f
	2.68-2.55 (2H, m, H-8 1)	4.6	c
	2.22 (3H, s, H-7 1)	1.7	d
2.84*	3.97-3.89 (1H, m, H-1)	6.2	n
	2.41 (1H, dd, $J = 14.4$, $J = 9.5$, $H_{\text{A}}-20$)	14.8	Not shown
	1.94 (3H, s, H-2 1)	2.1	p
	1.91 (3H, s, H-18 1)	Small	m
2.74*	4.88 (1H, s, H-15)	7.8	h
	2.68-2.55 (1H, m, $H_{\text{A}}-17^1$)	4.2	Not shown
	2.36-2.26 (2H, m, H-17 2)	4.8	i
	1.91 (3H, s, H-18 1)	Small	k
2.61	3.92 (2H, s, H-10)	1.9	e
	3.48-3.42 (2H, m, H-8 2)	4.9	c
	2.80-2.68 (1H, m, $H_{\text{B}}-17^1$)	3.4	Not shown
	2.36-2.26 (2H, m, H-17 2)	1.6	i
	2.22 (3H, s, H-7 1)	1.9	b
	1.91 (3H, s, H-18 1)	Small	k
2.41*	3.97-3.89 (1H, m, H-1)	8.6	n
	2.84 (1H, dd, $J = 14.2$, $J = 4.9$, $H_{\text{B}}-20$)	13.9	Not shown
	1.91 (3H, s, H-18 1)	2.2	m
2.31*	4.88 (1H, s, H-15)	1.0	j
	2.80-2.68 (1H, m, $H_{\text{B}}-17^1$)	1.9	i
	2.68-2.55 (1H, m, $H_{\text{A}}-17^1$)	1.9	i
	1.91 (3H, s, H-18 1)	1.0	l
2.22	9.31 (1H, s, H-5)	2.6	a
	3.48-3.42 (2H, m, H-8 2)	Small	d
	2.68-2.55 (2H, m, H-8 1)	Small	b
2.08	3.92 (2H, s, H-10)	Small	g
1.94*	6.43 (1H, dd, $J = 17.6$, $J = 11.7$, H-3 1)	2.2	q
	3.97-3.89 (1H, m, H-1)	1.4	o
	2.84 (1H, dd, $J = 14.2$, $J = 4.9$, $H_{\text{B}}-20$)	Small	p

* The neighbouring signals are affected by off-resonance irradiation. The reported values are referred to 100% intensity of the sum of the saturated signals.

† The other resonance signals were assigned on the basis of their multiplicities and chemical shifts: δ 3.73 (3H, s, H-13 3); 6.09 (1H, dd, $J = 17.6$, $J = 2.4$, $H_{\text{trans}}-3^2$).

the acidic proton at C(13 2) rapidly exchange H/D in CD_3OD . Accordingly, no coupling between protons on C(15) and C(13 2) could be observed. In $\text{DMSO}-d_6$, as expected, the coupling ($J = 3.4$ Hz) has been established. Nevertheless, the latter spectrum was unsuitable for NOE measurements, because of serious signal overlappings. The longest wavelength in UV/visible spectrum at 312 nm excluded an extended conjugational system [12], but agreed with the presence of an α -formylpyrrole [13]. The absorption spectrum is almost identical with the spectrum published for **3a** by Kräutler *et al.* [5]. HR-mass spectrometry shows a molecular ion at m/z 667 $[\text{M} + \text{H}]^+$ and established the exact molecular mass to be 667.2740 ± 0.0004 , appropriate

for $[\text{C}_{35}\text{H}_{40}\text{N}_4\text{O}_8\text{Na}]^+$ (calcd 667.2743) which is consistent with the NMR data for the constitution of sodium 3 1 ,3 2 -didehydro-1,10,15,20,22,24-hexahydro-8 2 -hydroxy-13 2 -methoxycarbonyl-4,5-dioxo-4,5-seco-21H,23H-phytoporphyrinate (**1**).

Neither the absolute nor the relative configurations of the three stereogenic centres at positions C(1), C(13 2) and C(15) are known; suitable crystals for X-ray structure analysis are not yet available. Nevertheless, the ease of H/D exchange on C(13 2) in ^1H NMR suggests that the small satellite peaks, for example at δ 2.00 and δ 9.27, originate from an equilibration of epimers in a ratio of 88:12. In ^{13}C NMR spectra, deuterium exchange at C(13 2) is also responsible for

the multiplet structure of the resonance found at δ 67.8 ppm. As already discussed for **3a** (see [4, 5]) the anti-configuration for the hydrogen atoms attached to C(13²) and C(15) is assumed to be the predominant epimer, owing to steric hindrance between the A/D half of the tetrapyrrolic moiety and the ester group on C(13²).

Signal splitting due to diastereomeric mixtures at the stereogenic centres C(1), C(15) have not been observed by NMR. In contrast, the syn- and anti-diastereomers of analogue structures obtained from the main red chlorophyll degradation product **2c** of *C. protothecoides* by partial synthesis could be separated by preparative TLC. Both isolated diastereomers exhibit in ¹H NMR distinct differences in chemical shifts, and were found to be stable at the chiral centres [1]. The chiral centre C(1) is expected to racemize under alkaline conditions, as has been convincingly demonstrated in a series of structurally related 5(2H)-dipyrromethanones [14]. Consequently, strong alkaline conditions should be avoided during the isolation procedure. Compound **1** shows optical activity in solution and a well-structured circular dichroism spectrum, exhibiting several Cotton effects (Fig. 3). The curve is almost identical to the CD spectrum of the catabolite isolated from *H. vulgare* [5]. This agreement suggests that the chiral centres are equally configured, implying the presence of a set of similar enzymes in different orders of angiosperms.

The molecular structure **1** of the catabolite isolated from naturally aged *C. japonicum* leaves suggests the following catabolic processes: (i) oxygenolytic ring opening at the *meso*-position with simultaneous protonation of the methine-bridge C(10), (ii) saturation of the remaining methine bridge on C(1)-C(20), (iii) aromatization of the original pyrroline ring D by proton

catalysed rearrangement and (iv) β -hydroxylation on C(8²). The basic constitution is closely related to the structures of the catabolites isolated from *H. vulgare* and *B. napus* compounds **3a** and **3b–3d**, respectively (Fig. 1). An intact methoxycarbonyl group is present at the isocyclic ring E in the catabolite of *C. japonicum* and *H. vulgare*, suggesting that oxidative ring opening precedes methyl ester cleavage. Experiments with nonspecific esterases (hog liver esterase or lipase from *Candida cylindracea*), which had previously been applied for the methyl ester cleavage of the propionic side chain [15], failed to hydrolyse the C(13²) ester group of compound **1**, implicating the requirement for a specific hydrolytic enzyme. All catabolites isolated to date from higher plants indicate the action of a specific enzyme(s), hydroxylating the nonactivated C(8²) methyl group (Fig. 1), which is absent or inactive in the green alga *C. protothecoides*. This hydroxylation step increases the polarity of the catabolite, thereby facilitating the relocation of the catabolites into the vacuoles of the senescent plant cells [16]. No catabolites of chlorophyll *b* were observed in the extract from *C. japonicum* or found in *H. vulgare* and *B. napus*. Hence, it remains uncertain whether chlorophyll *b*, as suggested, is enzymatically converted in higher plants into chlorophyll *a* prior to ring opening [17, 18] or whether the chlorophyll *b* catabolite decomposes and/or eludes detection.

Degradation of the chlorophylls in plants serves presumably to avoid the accumulation of photodynamic active tetrapyrroles by interrupting the chromophoric system during the ageing process in which nutrients are relocated [19, 20]. However, 233 mg of a yellowish nonfluorescent compound were isolated from 250 g (fresh-weight) leaves of *C. japonicum*. The amounts

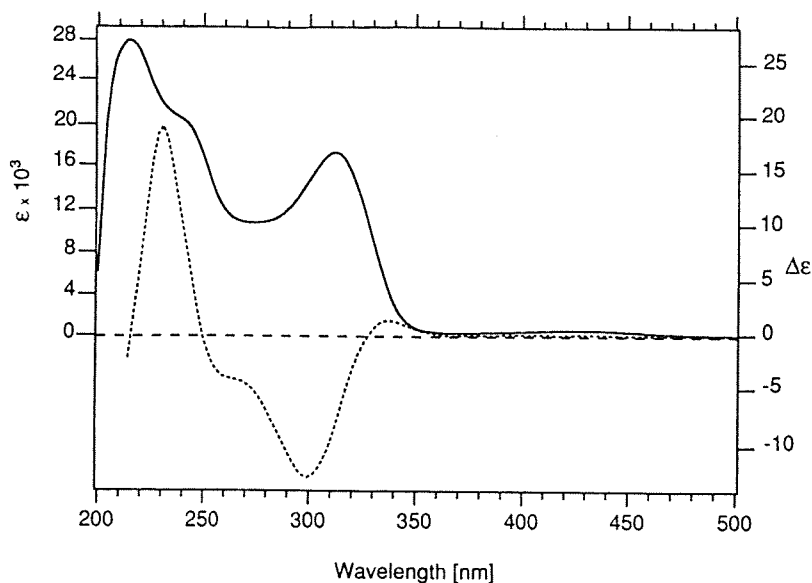


Fig. 3. Superimposed UV/visible (—) [$\epsilon = f(\lambda)$] and CD (····) [$\Delta\epsilon = f(\lambda)$] spectrum of the purified catabolite **1** in MeOH solution.

isolated agree quite well with the estimated content of 0.1–0.2% chlorophyll *a* generally found in fresh leaves of land plants [21], suggesting that chlorophyll catabolites are not further reutilized by the plant itself but rather (bio)degraded in the environment after leaf fall. Recently and independently from our work, Iturraspe *et al.* [22] reported the isolation of a structurally identical chlorophyll *a* catabolite from senescent leaves of *Liquidambar styraciflua* and *L. orientalis*. However, the chiroptical properties of this compound have not been reported.

Catabolism is generally associated with a series of chaotic destructive reactions under little control. Actually, the structures of the chlorophyll catabolites isolated from such diverse plant taxa as the Chlorophyceae, Gramineae, Cruciferae and Cercidiphyllaceae indicate a highly stereo-controlled process during catabolism, pointing to an orderly mechanism tightly controlled by enzymes.

EXPERIMENTAL

Materials. All chemicals were reagent grade; solvents were distilled prior to use. TLC aluminium foils precoated with silica gel 60 PF₂₅₄ (0.2 mm) and preparative TLC plates coated with silica gel 60 PF_{254–366} (1.25 mm thick, 20 × 20 cm) were purchased from Merck.

Spectra. ¹H NMR: 360.14 MHz with a Bruker AM-360 instrument supplied with an Aspect 3000 data system. Chemical shifts (δ) are given in ppm downfield from TMS, as int. standard, and coupling constants (J) in Hertz. ¹³C NMR spectra were measured at 125.76 MHz with a Bruker Avance DRX-500 instrument using CD₃OD as int. standard (δ 49.0). MS and HR-MS were obtained with a Vacuum Generator Micromass 7070 E instrument equipped with a DS 11-250 data system using the FAB ionization in positive mode with Xe as primary atom beam at 7 keV and 1 μ A. Samples were first dissolved in methanol (MeOH) and then added to the glycerol matrix. Accurate masses were obtained by internal matching with nearby matrix signals. Quantitative sodium-ion determination was performed by flame emission on a Video 12 spectrophotometer. NaI dissolved in MeOH was used for calibration, emissions were observed at 589 nm. UV/VIS spectra were recorded with a Hewlett Packard 8452A diode-array spectrophotometer. CD spectra were obtained with a Jobin-Yvon Auto Dichograph Mark V; $\Delta\epsilon$ [l mole⁻¹ cm⁻¹]. Polarimetric measurements on a Perkin Elmer 241 MC instrument.

Plant material. Fresh yellow leaves were collected in September 1995 from the branches of a single male tree of *C. japonicum* Sieb. et Zucc., cultivated at the Botanical Garden Fribourg, Switzerland.

Detection of the catabolites. In order to detect colourless chlorophyll catabolites the 'on spot chromic acid degradation' technique was used, basically performed as suggested in [23], where bile-pigments are oxidized directly on TLC plates with chromic acid to

the corresponding succinimides and/or maleimides. Accordingly, a spot of the CH₂Cl₂ extract obtained from the leaves of *C. japonicum* was overlaid with chromic acid soln. After developing the TLC plate in CH₂Cl₂–EtOAc–EtOH–HOAc (100:20:10:1) and staining, the presence of a pyrrolic compound was indicated by the appearance of blue-coloured spots. The position of the catabolite after developing of the spot on TLC in the first dimension using solvent systems of different polarities was found by application of the above procedure to the entire vertical lane. The corresponding spot, when irradiated at 254 nm, showed quenching of the fluorescent silica gel layer and was eventually isolated by prep. TLC. The purified compound assumed a rusty-like colour on the TLC plate when exposed to air and light after about two hr, as observed in [16] for the catabolite isolated from *H. vulgare*.

Isolation. 250 g (fresh wt) leaves of *C. japonicum* were mixed for 30 sec in a Waring Blender Model 32BL79 in a soln of Me₂CO–MeOH (1:1) (750 ml) at high speed without cooling. After centrifugation of the resulting slurry for 5 min at 5500 g, the pellets were washed with the same solvent mixt. (960 ml). After centrifugation, the combined supernatants were immediately filtered, concd to 250 ml by evapn of the solvents *in vacuo*, and then diluted with water (1000 ml). Extraction of the slightly turbid soln exhibiting pH 3 with CH₂Cl₂ (5 × 250 ml), drying of the extract with Na₂SO₄ and evapn of the solvent *in vacuo* yielded a residue which was filtered over a short chromatography column (9 cm high, 5 cm diameter, containing silica gel 40–63 μ m) and eluted with CH₂Cl₂–MeOH (4:1). The collected frs were concd *in vacuo* and applied to 10 prep. TLC plates which were subsequently developed in CHCl₃–MeOH–H₂O (80:15:2). Extracting of the silica gel zone containing the catabolite with MeOH afforded after evapn of the solvent *in vacuo* a residue which was dissolved in MeOH–CH₂Cl₂ (1:1) (10 ml) and filtered over a degreased cotton plug. Evapn of the solvent *in vacuo* and drying of the solid in high vacuum at room temp. yielded 233 mg of a non-fluorescent, slightly yellow compound which was directly used for spectroscopic analysis. UV/VIS: $\lambda_{\max}^{\text{MeOH}}$ nm (log ϵ): 214 (4.44), 244 sh (4.29), 312 (4.23). CD: $\lambda_{\max}^{\text{MeOH}}$ nm ($\Delta\epsilon$): 227 (+19.7), 252 sh (–4.0), 285 (–13.5), 316 (+1.6) (MeOH; c 4.80 × 10⁻² mM). $[\alpha]_D^{20} = -152 \pm 1^\circ$ (MeOH; c 0.0785). ¹³C-DEPT-NMR (distortionless enhancement by polarization transfer (DEPT)) (CD₃OD): 8.9, 9.3 and 9.4 (C-2¹, C-7¹ and C-18¹), 12.5 (C-12¹), 22.5 (C-17¹), 23.9 (C-10), 28.1 (C-8¹), 30.6 (C-20), 37.3 (C-15), 40.5 (C-17²), 52.9 (C-13⁵), 62.2 (C-1), 62.7 (C-8²), 67.8 (m, C-13²) (multiplet due to H/D exchange with the solvent), 119.1 (C-3²), 127.2 (C-3¹), 112.2, 115.6, 2 × 120.9, 124.4, 124.8, 125.8, 128.8, 129.4, 134.3, 135.5, 139.2, 156.9 and 161.8 (14 × C_{quat}), 171.9, 174.7 and 182.8 (lactam CO, ester COO and COO), 177.8 (CHO), 192.0 (CO). FAB-MS: m/z (%): 645 (17, [M + 2H – Na]⁺), 667 (100, [M +

H]⁺, 689 (56, [M + Na]⁺). $R_f = 0.46$; CHCl₃-MeOH-H₂O (v/v/v, 80:15:2). ¹H NMR: Table 1.

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New Trends in Photobiology (Invited Review)

Chlorophyll catabolism – structures, mechanisms, conversions

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Abstract

Several chlorophyll catabolites have been isolated and characterised from higher plants (angiosperms) and from a green algae (chlorophyte) during the last five years. A common feature of all chlorophyll catabolites isolated thus far is the unexpected regioselectivity of the oxidative cleavage of the macrocycle at the C(5)-methene bridge, yielding bile-pigment-like 19-formyl-1[21H,23H]bilinones. Recent in vivo $^{18,18}\text{O}_2$ -labelling experiments performed with the green algae *Chlorella protothecoides* showed unequivocally that a mono-oxygenase is involved in the primary oxidative cleavage step of the chlorophyll macrocycle. Moreover, the main red chlorophyll *a* catabolite from *C. protothecoides* has been chemically converted into the skeleton of the colourless catabolites isolated from angiosperms. On this basis, a likely catabolic pathway is outlined, which comprises all the products of chlorophyll breakdown isolated until now from both a chlorophyte and from higher plants.

Keywords: Chlorophyll catabolism in green algae; *Chlorella protothecoides*; Chlorophyll catabolites in a chlorophyte; Chlorophyll catabolites in angiosperms; Autumnal chlorophyll degradation; Linear tetrapyrroles

1. Introduction

It is surprising that one of the most spectacular recurrent phenomena on earth, the fascinating change of colour of leaves at the beginning of the autumn, has only sporadically deserved the attention of chemists in past decades. Incipient systematic studies on chlorophyll degradation in plants were carried out at the beginning of this century. Separation of pigments contained in leaves by column chromatography, which was invented by Michail Cvet (Tswett) between 1906 and 1908, allowed to ascertain that the yellow colour of autumn leaves is due to carotenoids (mainly lutein = xanthophyll) which are already present in the plant cells before their loss of greenness, and are not related, therefore, to the breakdown of chlorophyll. Moreover, although at this time the structure of chlorophyll was unknown (it was elucidated by Hans Fischer and his school in 1940) a transformation of chlorophyll into carotenoids could be also excluded [1]. Interestingly, this speculation was reconsidered in 1968 as a pathway for the biogenesis of secondary carotenoids in some green algae [2]. Indeed, the fact that chlorophyll disappears from wilting leaves without leaving a trace has intrigued plant physiologists for over nine decades [3].

2. Chlorophyll degradation: a common process in plant development

Although ageing or senescence of leaves of deciduous plants is the most manifest of the processes in which fundamental changes of the structure of the chlorophyll molecule occur, there are several other biological processes in plants in which chlorophyll is apparently destroyed. Thus, evidence for a continuous degradation and biosynthesis (i.e. turnover) of chlorophylls in healthy plants is being increasingly well documented. Moreover, during seed germination, flowering, and fruit ripening as well as when green plants are deprived of light (so-called bleaching), the resulting decrease in chlorophyll content can be regarded as an ageing process, although the involved mechanisms of chlorophyll degradation must not necessarily be the same in all cases. Moreover, in most plant species, the expansion of the first true leaves after germination results in senescence of the cotyledons with concomitant loss of chlorophyll. During flowering and fruit ripening, the development of proplastids to chloroplasts is branched off to the formation of chromoplasts, which usually contain much more carotenoids than chlorophylls. Bleaching of green plants is induced most frequently by the lack of appropriate illumination conditions. In some unicellular algae

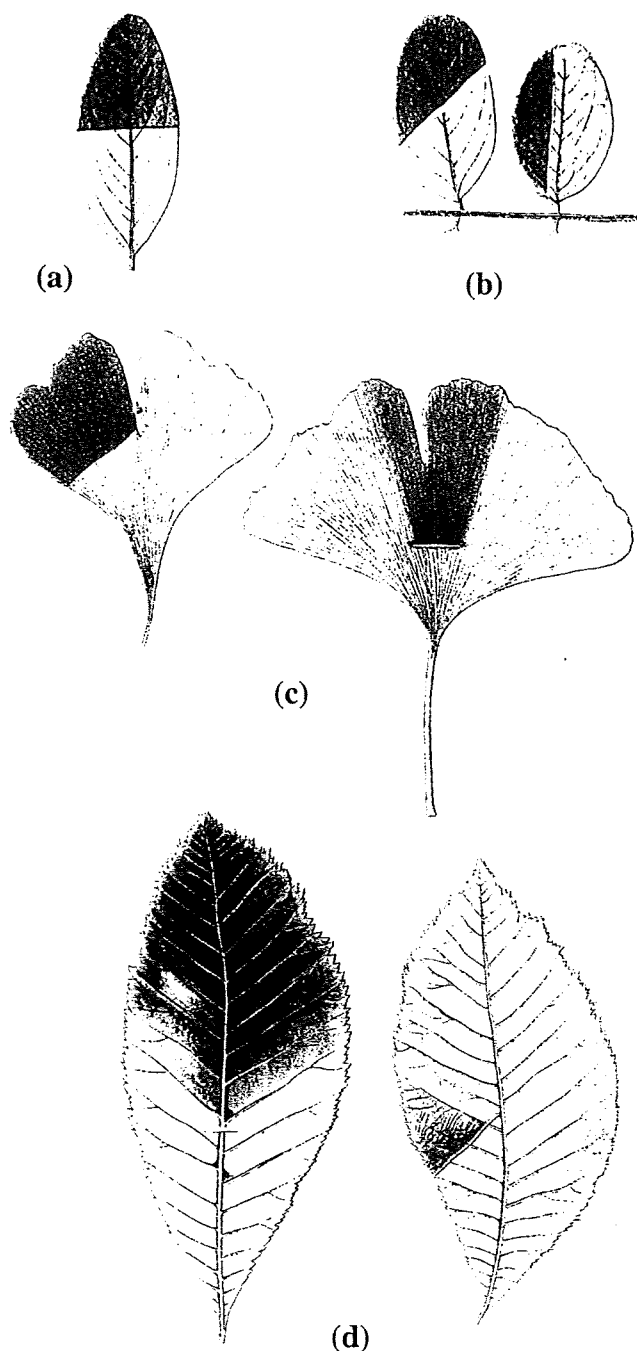


Fig. 1. Effects of different kinds of injury of the leaf blade at the beginning of loss of greenness of various plant species (taken from E. Stahl [13]). The leaf turns yellow normally beneath the position of folding, incision or cutting of the leaves, whereas the parts of the blade above the incisions remain green for much longer. (a) *Robinia viscosa*, after folding of the upper half of the leaf blade; (b) *Robinia pseudaccacia* after dissection of the leaf blade; (c) *Ginkgo biloba* after folding or incision across the leaf veins; (d) *Aesculus rubicunda* after incision of the main vein or placing a half-side cut into the branches of the veins.

chlorophyll is associated in the chloroplasts, so that most likely, a complete degradation of the chlorophyll during the loss of greenness of leaves does not take place. More recent investigations of the chlorophyll catabolism in phanerogams

suggest that they degrade chlorophyll because the senescent cells must eliminate dangerous photodynamic molecules which are liberated upon the mobilisation of the corresponding apoproteins. Detoxification is necessary because orderly metabolism can take place only if the senescent cells are intact and compartmentalised [19].

5. A brief outline on the nomenclature of chlorophylls

The present review aims to resume recent progress done on the study of chlorophyll catabolism; literature prior to 1987 has been comprehensively compiled by Hendry et al. and Brown et al. [3]. It seems to be suitable however, to summarize here the most important features of the chlorophyll molecule in order to familiarize the reader with the somewhat intricate terminology on this field.

The name chlorophyll (gr. $\chi\lambda\omega\rho\acute{o}\varsigma$ = green, $\phi\acute{\upsilon}\lambda\lambda\omicron\nu$ = leaf) was given to the green pigment in leaves by Pierre Joseph Pelletier and Joseph Bienaimé Caventou in 1818. Only almost one hundred years later, however, was the study of this interesting molecule settled on sound scientific ground by Richard Willstätter, who obtained for the first time pure chlorophyll and established its correct molecular formula. In 1911, Willstätter originally gave chlorophyll the molecular formula $C_{55}H_{72}MgN_4O_6$, but one year later he showed that chlorophyll, obtained from a wide variety of sources, was a mixture of two compounds, chlorophyll *a* and chlorophyll *b*, to which he assigned the correct molecular formulae $C_{55}H_{72}MgN_4O_5$ and $C_{55}H_{70}MgN_4O_6$ respectively. Willstätter also described the enzyme chlorophyllase, which cleaves phytol from both chlorophylls yielding chlorophyllide *a* and *b* respectively [20] (cf. Scheme 1). The molecular structures of chlorophyll *a* (1a) and *b* (1b) were established by Hans Fischer in 1940. The structure of chlorophyll *a* was confirmed by total synthesis in Woodward's laboratories in 1960 [21]. Thereafter, the correct absolute configuration at the asymmetric atoms C(17) and C(18) of the molecule was established by Flemming [22] and Brockmann [23].

Treatment of chlorophylls under acidic conditions causes demetallation, affording grey-brown pheophytin (gr. $\varphi\alpha\iota\acute{o}\varsigma$ = brown) *a* and *b*, which on hydrolysis yields pheophorbide *a* (3a) and *b* (3b) respectively. When pheophorbide *a* or *b* is heated under reflux in collidone for 90 min, the corresponding pyropheophorbides (4a and 4b) are obtained (Scheme 1).

According to IUPAC-IUB recommendations [24,25], the incomplete Fischer numeration (cf. formula 5a (Fig. 2)), in which the peripheric positions of the porphyrin macrocycle are numbered from 1 to 8 and the methene bridges between the pyrrole rings are designated with the four first letters of the Greek alphabet, has been abandoned for the porphyrin nucleus, and the 1–24 numbering scheme depicted on formula 5b is adopted for all tetrapyrroles. In the present work, the atoms of side chains at the porphyrin nucleus are numbered starting at the first atom bound to the macrocycle, which is

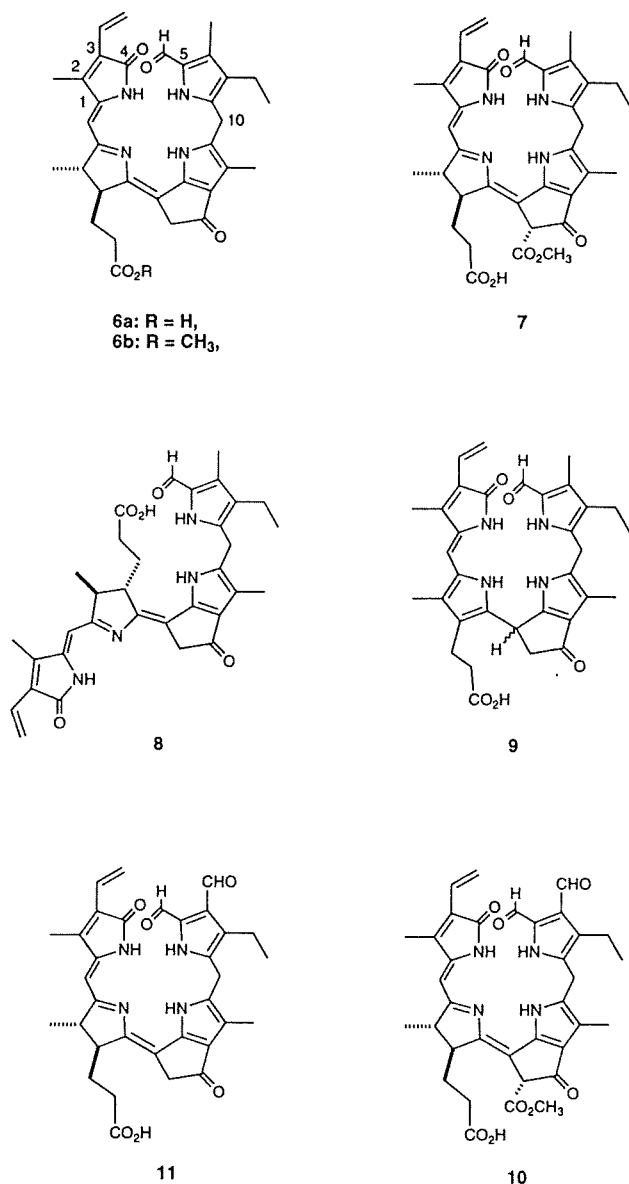


Fig. 3. Characterised tetrapyrrolic chlorophyll *a* and *b* degradation products isolated from *Chlorella protothecoides* after acidification of the culture medium. Compound 6b was obtained after esterification of 10,22-dihydro-4,5-dioxo-4,5-seco-21H,23H-pyropheophorbide *a* (6a) with methanol.

inated in the present work as seco derivatives of the latter, whereby according to IUPAC rule R-1.2.6.2 [27], the prefix “seco” indicates cleavage of the ring with addition of one or more hydrogen atoms at each terminal group thus created (*cf.* Fig. 3).

6. Enzymatic breakdown of chlorophyll

When dealing with chlorophyll catabolism, it is useful to differentiate between reactions which do not change the fundamental structure of the chromophore, such as loss of magnesium, hydrolysis of the phytol residue, hydroxylation at C(13²) (allomerization reaction), or demethoxycarbonyla-

tion at C(13²) and the proper degradation of the chlorophyll, i.e. the cleavage of the macrocycle. The first types of reaction may precede or follow the degradation of the macrocycle, in a sequence which is as yet unknown.

To date, two enzymes, which may be involved in the process of chlorophyll catabolism, namely chlorophyllase and magnesium dechelatease, have been characterised in plants. Chlorophyllase catalyses both the formation of the phytol ester bond as well as its hydrolysis (*vide supra*) and has been implicated many times in the past as part of a degradative mechanism [28,29]. The enzyme, which is localised in chloroplast membranes, has been solubilised and purified from several sources. Thus, chlorophyllase from the diatom *Phaeodactylum tricornutum* is a glycoprotein consisting of 30 kDa subunits [30,31]. The enzyme from rye seedlings has a molecular weight of 39 kDa [32]. The enzyme activity is enhanced by membrane lipids and Mg ions [33]. The regulation of chlorophyllase activity is puzzling, since the breakdown of chlorophyll depends on the cytoplasmatic protein biosynthesis though the enzyme is already present in the thylakoids [34].

Since all products of the enzymatic ring cleavage of chlorophyll which have been characterised so far lack the phytol residue (*vide infra*), chlorophyllase is most likely to be involved in chlorophyll catabolism. Interestingly, gerontoplasts accumulate phytol acetate in the plastoglobuli during leaf senescence [35].

Enzymes responsible for the removal of the central chelated magnesium ion, so-called magnesium-dechelateases, have also been reported on a number of occasions. However, none of the systems investigated in the past has been fully characterised, partly because of the difficulty of distinguishing endogeneous enzymatic demetallation from artifacts. The presence of a Mg(II)-dechelatase in senescent chloroplasts has been demonstrated by Matile et al. [36], so that it may be anticipated that loss of the magnesium atom precedes the oxidative cleavage of the macrocycle in the enzymatic degradation process. More recently, an enzyme catalysing the conversion of pheophorbide *a* to a pyropheophorbide *a* precursor has been found in the soluble proteins fraction of *Chenopodium alba*. The precursor was subsequently converted non-enzymatically to pyropheophorbide *a* [37].

However, although the occurrence of a chlorophyll oxidase in barley thylakoids has been reported [38–40], none of the products of enzymatic ring cleavage of chlorophyll, which have been characterised since (see Section 7), are derived from 13²-hydroxychlorophyll, so that the latter is probably an artefact produced by autoxidation (so-called allomerization [41]) of chlorophyll under the conditions of experimental senescence [40]. Recent work on chlorophyll catabolism proves unequivocally that an enzymatic oxidation step is involved in the cleavage of the macrocycle [42,43]. This is substantiated by the elucidation of the structures of the products of *in vivo* breakdown of chlorophyll in presence of ¹⁸O-labelled dioxygen (*cf.* Sections 7 and 8 respectively). In barley, the oxidising enzyme requires ferredoxin for the oper-

ation of its redox cycle [44]. Interestingly, the formation of the (as yet uncharacterised) primary product of chlorophyll catabolism produced in dark bleached barley cotyledons consumes ATP [45].

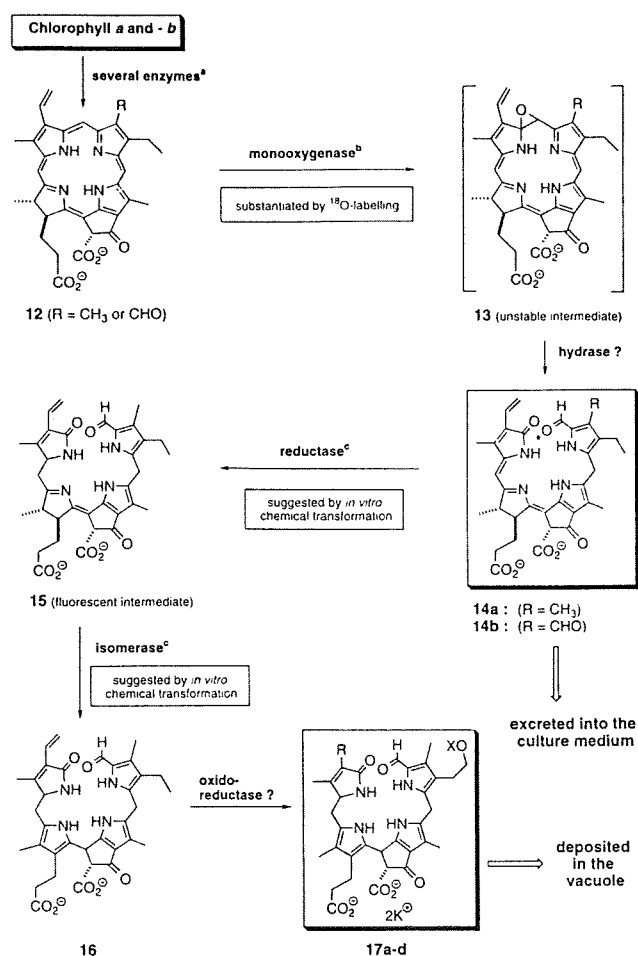
A further unanswered question is whether the chlorophyll which is degraded in living cells is still present as a pigment–protein complex or not. Several attempts have been made to examine chlorophyll breakdown where the substrate is in association with both protein and lipid [46–48]. Although from these studies, it seems highly probable that there are distinct degradative reactions which take place within the lipid environment of the thylakoids and which utilize chlorophyll bound to one or more proteins as a substrate, much of the work done before the actual structures of the products of chlorophyll catabolism were known should be repeated considering the now available experimental evidence.

In the older literature, light and oxygen were considered as essential for the degradation of the chlorophyll macrocycle in living organisms [3]. From the present point of view, this dogma must be called into question since enzymatic degradation of chlorophyll in bleaching cells of the green algae *Chlorella protothecoides* and of the primary leaves of barley takes place also in darkness [49,50]. Otherwise, all hypotheses formulated prior to 1991 in connection with the possible mechanisms of chlorophyll breakdown must be regarded as speculative.

7. The molecular structures of primary chlorophyll catabolites

In 1991 two Swiss research groups succeeded for the first time, independently of each other, in the isolation and characterisation of tetrapyrrolic chlorophyll *a* degradation products from dark-bleached excised primary leaves of barley (*Hordeum vulgare* cv. Gerbel) [50,51] and from the culture medium of the microalgae *Chlorella protothecoides*, which had been grown in a medium rich in glucose but poor in nitrogen sources [49]. Shortly afterwards, the structure of the pigment **6a** (cf. Fig. 3), isolated from the latter, was confirmed by X-ray diffraction analysis [52], as well as by partial synthesis starting from pyropheophorbide *a* [53]. More recently, similar chlorophyll *a* catabolites (**17b–17d**) have been isolated from senescent rape cotyledons [54,55] (cf. Scheme 2).

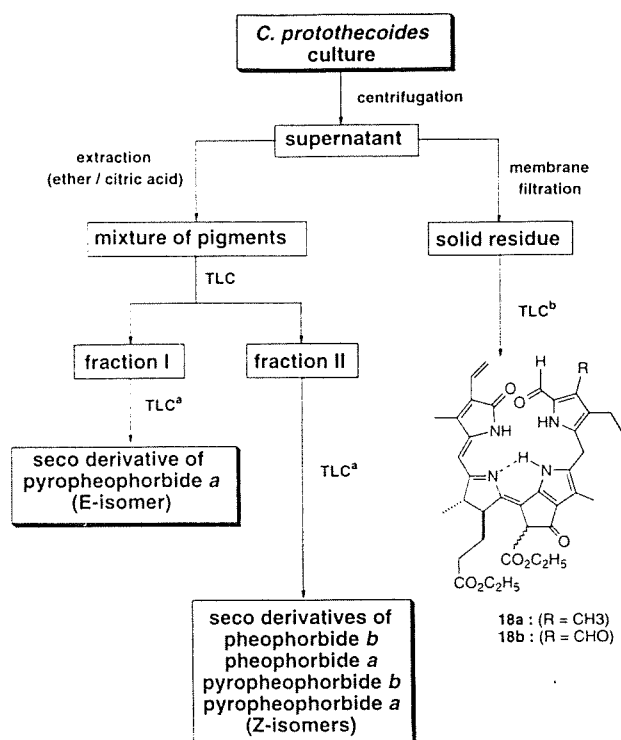
Against earlier mechanistic speculations, the common feature of all chlorophyll degradation products, which have been isolated hitherto from a green algae (*Chlorella*) as well as from a monocot (barley) and a dicot (rape), is the unexpected regioselectivity of the oxidative cleavage of the macrocycle at the C(5)-methene bridge instead of at the double bonds adjacent to the reduced pyrrole ring, as generally believed before [3]. However, although the regioselectivity of the cleavage of the chlorophyll macrocycle is formally reminiscent from that of haeme catabolism [56], it must be pointed out that the product of ring cleavage of the



Scheme 2. Suggested pathway of the enzymatic degradation of chlorophylls. The probable relationship between the catabolites excreted into the culture medium by bleaching *Chlorella protothecoides* cells and those isolated from barley leaves and rape cotyledons is shown. The structures of the compounds which have been definitively recognised hitherto as chlorophyll catabolites are framed. ^a Presumably consecutive action of chlorophyllase, magnesium dechelatase and hydrolase [3]. ^b Substantiated by ¹⁸O-labelling [72]. In formula **14a** the oxygen atoms proceeding from water and molecular oxygen (*O) are differentiated by an asterisk. ^c Suggested by *in vitro* chemical transformation. **17a**: X=H, R=1, 2 dihydroxyethyl, as O(13⁺) methylester in barley [50]; **17b**: X=H, R=vinylyl, in rape [55]; **17c**: X=malonyl K⁺ salt, R=vinylyl [54], **17d**: X=1-glucosidyl, R=vinylyl, in rape [55].

former is not a bile pigment but a 19-formyl-1[21H,22H]bilinone derivative, i.e. no carbon atom is lost during the oxidative opening of the chlorophyll macrocycle. Moreover, whereas three oxygen molecules are involved in the oxidative ring cleavage of haeme [57], only one oxygen molecule is required for the formation of **6a** (cf. Section 8).

In contrast to the pigments excreted into the culture medium by *Chlorella* cells, the chlorophyll catabolites isolated by Matile et al. from phanerogams are colourless compounds, which turn to a rusty colour on exposure to air on silica-gel plates [35]. Thus, it becomes clear why the primary degradation products of chlorophyll have been overlooked for such a long time. However, the presently available results point to the fact that chlorophyll catabolism may involve several reductive steps which are absent in the deg-



Scheme 3. Schematic representation of the isolation procedures used for the separation of the mixture of pigments contained in the culture medium of bleached *Chlorella protothecoides* cells. ^a Separation of the methylester by thin-layer chromatography (TLC) after esterification with MeOH [49,62,78]. ^b Separation of the diethylester by TLC after esterification with EtOH/BOP/Et₃N [61]; BOP, benzotriazol-1-yloxy tris(dimethylamino)phosphonium hexafluorophosphate.

radation of haeme, disregarding the reduction of biliverdin to bilirubin, which takes place only in mammalia. As it is well documented that polypyrrolic compounds in which the pyrrole rings are joined by methylene bridges are readily cleaved by protolysis [58], a further degradation of compounds like **17a–17d** should be a rapid process. Until now however, the fate of the products formed after cleavage of the chlorophyll macrocycle is not known.

As mentioned above, the first chlorophyll degradation products isolated from the culture medium of bleaching *Chlorella protothecoides* cells have been characterised as derivatives of pyropheophorbide. Consequently, enzymatic demethoxycarbonylation at C(13²) was assumed to occur [59], supported indeed by the fact that pyropheophytin and pyropheophorbide had been found earlier in photosynthetic organisms [60]. Most recently, however, the isolation procedure used to isolate the pigments present in the culture medium of *C. protothecoides* has been modified in that the separated nutrient broth is simply filtered through a membrane filter and the solid residue is chromatographed on silica–gel after esterification with ethanol. By this procedure, only two pigments have been obtained, namely the 19-formyl-1[21H,22H]bilinone derivatives **18a** and **18b**, which originate from chlorophyll *a* and *b* respectively [61] (cf. Scheme 3). As both derivatives are isolated as their ethyl

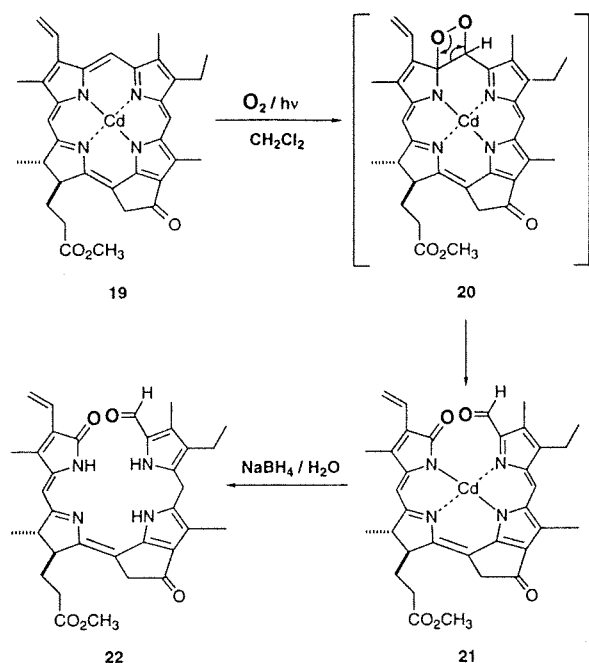
esters, it must be concluded that the pigments originally present in the culture medium are the corresponding catabolites of O(13⁴)-demethylpheophorbide *a* and *b*, which have to be regarded, therefore, as the primary products of oxidative breakdown of the chlorophyll in *C. protothecoides* (vide infra). Accordingly, the chlorophyll *a* catabolite isolated from senescent rape cotyledons has been characterised as the potassium salt of a O(13⁴)-demethylphytytoporphyrin *a* derivative [54].

Until now, degradation products of chlorophyll *b* (i.e. **10** and **11**) have been isolated only from the culture medium of bleaching *Chlorella protothecoides* cells [62]. Surprisingly, chlorophyll *b* catabolites are absent from both dark-bleached primary leaves of barley and senescent rape cotyledons [63]. This finding may be explained by the recently discovered transformation of chlorophyll *b* into chlorophyll *a* [64–66], which may take place to a large extent before degradation. Earlier studies [67,68], which claim a decrease of the ratio of chlorophyll *a* to chlorophyll *b* at the beginning of senescence are, however, in disagreement with this hypothesis.

8. A tentative mechanism of enzymatic chlorophyll breakdown

A first insight into the mechanism of enzymatic degradation of chlorophyll was furnished by the discovery that photo-oxidation of pyropheophorbide metal chelates in organic solvents yields similar products as those isolated from the culture medium of *C. protothecoides* [69]; the regioselectivity of the ring cleavage being dependent on the complexed metal ion [70]. Actually, an investigation of the photo-oxidative ring cleavage of the Cd(II)-chelate of pyropheophorbide *a* in vitro and of the enzymatic transformation of chlorophyll *a* into **6a**, both in the presence of ¹⁸O₂, revealed that the transformations proceed by different mechanisms. As a matter of fact, the pattern observed in the range of the molecular ion peaks of the photo-oxidation product, after reductive demetallation, agrees with the incorporation of two oxygen atoms proceeding from the same molecule. Most likely, therefore, the obtained formylbilinone derivative is formed via a dioxetane **20**, resulting from the cycloaddition of a singlet oxygen molecule to the C(4)=C(5)-bond of the substrate [71] (cf. Scheme 4). Conversely, after bleaching of *C. protothecoides* cells in the presence of ¹⁸O₂, it could be unequivocally demonstrated by mass spectrometric analysis of the red bilin derivative **25** excreted into the culture medium, that only the oxygen atom of the formyl group proceeds from molecular oxygen, whereas the oxygen atom of the lactam carbonyl originates from water [72] (cf. Scheme 5). Thus, an oxygenolytic cleavage of the chlorophyll macrocycle, as is presumed to operate in phanerogams [44], is in disagreement with the evidence gained from the aforementioned ¹⁸O-labelling experiments.

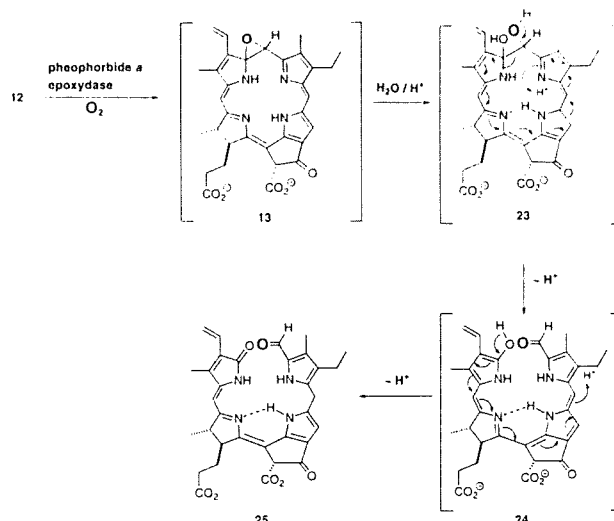
On the basis of the above results, a consistent (although in some details still hypothetical) catabolic pathway may be



Scheme 4. Photo-oxygenation of the Cd(II)-chelate of pyropheophorbide *a* (19) and subsequent reductive demetallation of the intermediate 21 affords the red pigment 12, identical with the natural catabolite isolated from the culture medium of bleaching *Chlorella protothecoides* cells. The fate of the oxygen molecule during the photo-oxidative process is indicated in bold atom labels.

outlined (*cf.* Scheme 2), which comprises all the products of chlorophyll breakdown isolated until now from both microalgae and angiosperms, as well as the probable structure of a common unstable intermediate 13, which is presumably formed by the action of a monooxygenase on pheophorbide derivative 12. Subsequent regioselective hydrolysis of the epoxide intermediate affords a diol 23 (*cf.* Scheme 5) which may be cleaved by a retroaldol-like reaction yielding, after prototropic rearrangement, the seco-derivative 6a as the final product. It must be pointed out that this mode of cleavage of the macrocycle 23 takes place without change of the oxidation level of the molecule. Of course, an alternative mechanism, in which the diol is oxidatively cleaved and the C(9)=C(10) bond is hydrogenated enzymatically cannot be ruled out at present. As chlorophytes are generally accepted to be the phylogenetic ancestors of green plants [73], it may be expected that the same degradation pathway holds true for all oxygenic phototrophs evolved from chlorophytes.

Accordingly, transformation of the final product of chlorophyll breakdown in *C. protothecoides* (6a) into the skeleton of the colourless catabolites isolated from both barley and rape leaves would involve a reduction step (at the C(20)-methene bridge) and two prototropic shifts. As a result of model experiments carried out *in vitro*, pigment 6b could be transformed into a tetrapyrrolic derivative with the same chromophore as the chlorophyll catabolites isolated from phanerogams (Scheme 6). Thus, catalytic hydrogenation of 6b followed by treatment with acetic acid at 118 °C affords 26 in an overall yield of 51% as a 56 to 44 mixture of dia-



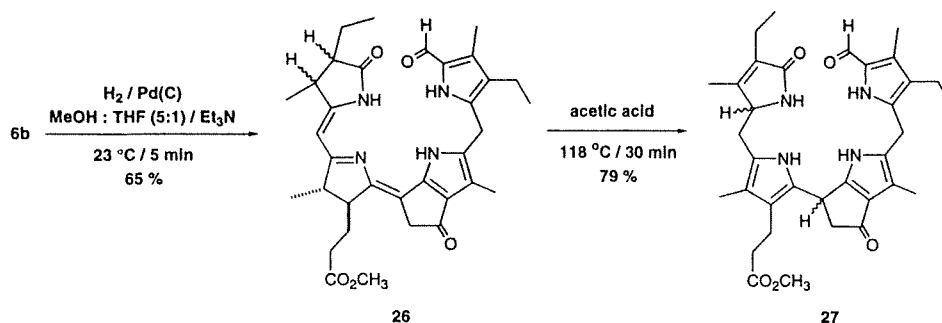
Scheme 5. Mechanism proposed for the ring cleavage of chlorophyll *a* involving the action of a mono-oxygenase in the key step. The following sequence for the ring opening is suggested: (i) addition of a monooxygenase-activated oxygen molecule to the C(4)=C(5) bond yields an oxirane derivative 13; (ii) regioselective cleavage of the oxirane ring by spontaneous or enzymatic hydrolysis results in the formation of an intermediate 22 in which the oxygen label remains bound to position C(5); (iii) retro-aldol condensation type cleaves the macrocycle; (iv) proton rearrangement eventually yields the isolated red chlorophyll *a* catabolite 25 [72].

stereomers. Moreover, intermediate 27, obtained upon catalytic hydrogenation of 6b, displays a noticeable fluorescence, which is reminiscent of that of some as yet uncharacterised chlorophyll catabolites in phanerogams [45,74–76] as well as of the chromophore of dinoflagellate luciferin [7]. It may be anticipated, therefore, that a 1,4,5,6-tetrahydro-5-methylene-4-oxo-cyclopenta[b]pyrrole subunit is responsible for the fluorescence in this series of compounds.

The final product 27 differs from the catabolites isolated from barley and rape leaves, 17a and 17d respectively, in the lack of the hydroxy substituents which are present at the ethyl groups of the latter (*cf.* Scheme 2). Whether enzymatic hydroxylation takes place before or after oxidative cleavage of the chlorin macrocycle is for the present not known. As *C. protothecoides* excretes the products of chlorophyll catabolism in the culture medium whereas the phanerogams, which have been investigated so far, accumulated them in the vacuoles of mesophyll cells [77], it may be speculated that the purpose of the enzymatic hydroxylation of the ethyl side chains of the catabolic pigments is to increase their solubility in water. Accordingly, the catabolites isolated from rape cotyledons are either esterified with malonic acid or they have been transformed into a glycoside [55].

9. Outlook

The catabolic pathway depicted in Scheme 2 should be considered as a working hypothesis, which needs to be corroborated in the near future by more *in vitro* experiments and,



Scheme 6. Catalytic hydrogenation of the red pigment 6b from *Chlorella protothecoides* followed by acid-catalysed rearrangement affords 27 which shows the same tetrapyrrolic skeleton as the colourless catabolites isolated from angiosperms (Scheme 2) [61].

ultimately, by the isolation of the different enzymes involved in the catabolism of chlorophylls. Nevertheless, our present knowledge of this process suggests a consistent picture, in which the evidence gained from taxonomic different living organisms by independent research groups is, until now, in significant good agreement.

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The authors are particularly indebted to the Swiss National Science Foundation for the generous financial support of our research projects related to chlorophyll catabolism. In the present review, a number of the authors' own contributions to the subject treated are reported. All coworkers, whose names are mentioned in the references, deserve our sincere thanks for their valuable help, encouraging enthusiasm, and fruitful experimental abilities. We are greatly indebted to Prof. H. Senger (Fachbereich Biologie/Botanik der Universität Marburg, Marburg/Lahn, Germany) for having drawn our attention to *Chlorella protothecoides* as well as for having provided us with cells of this strain for our initial experiments.

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Chlorophyll catabolism in *Chlorella protothecoides*. Part 8: Facts and artefacts ⁽¹⁾

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Abstract

O(13⁴)-demethyl-10,22-dihydro-4,5-dioxo-4,5-seco-21H,23H-pheophorbide *a* and *b* (**3a** and **3b**, respectively) are the primary products of cleavage of the chlorophyll macrocycle which are present in substantial amounts (up to 60% of the estimated initial chlorophyll content in the incubated cells) in the culture medium of bleaching *Chlorella protothecoides*. Other red tetrapyrrolic pigments previously isolated from the culture medium of this microalgae are formed by chemical transformation of **3a** and **3b**. Thus, as loss of the carboxy group at C(13²) takes place readily when the isolation of the pigments is carried out in acidic medium, the formation of the corresponding pyropheophorbide derivatives is most likely not an enzymatically catalysed step in chlorophyll catabolism. On the other hand, the main chlorophyll *a* degradation product **7a**, which had been isolated previously from *C. protothecoides* cultures, can be transformed chemically, in two "biomimetic" reaction steps, into a colourless tetrapyrrolic compound **12**, the structure of which corresponds (except for some peripheral substituents) to that of the chlorophyll *a* catabolites isolated from dark-bleached primary leaves of barley and from senescent rape cotyledons in other research groups. Thus, a common pathway may be suggested, as a working hypothesis, for the enzymatic degradation of chlorophylls in both chlorophytes and phanerogames.

Key words

Chlorophyll catabolism, *O*(13⁴)-demethylpheophorbides, 4,5-dioxo-4,5-seco-21H,23H-pyropheophorbides, 19-formyl-1[21H-22H]bilinones, *Chlorella protothecoides*.

Abbreviations

BOP, Castro's reagent: (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; CD, circular dichroism; CDI, 1,1-carbonyldiimidazole; DMSO, dimethyl sulfoxide; ee, enantiomeric excess; Et₃N, triethylamine; FAB, fast atom bombardement; MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; Phe-*a* and Phe-*b*, pheophorbide *a* and *b*, respectively; PPhe-*a* and PPhe-*b*, pyropheophorbide *a* and *b*, respectively; R(-)-ATFE, (R)(-)-1-(9-anthryl)-2,2,2-trifluoro ethanol; THF, tetrahydrofuran; TLC, thin-layer chromatography. Nomenclature of tetrapyrroles according to IUPAC-IUB (*Pure & Appl. Chem.*, **59**, 779-832, 1987).

INTRODUCTION

In 1991, two Swiss research groups succeeded for the first time, independently of each other, in the elucidation of the structures of tetrapyrrolic degradation products of chlorophyll *a* previously

isolated from the culture medium of bleaching *Chlorella protothecoides* cells (Engel *et al.*, 1991) and from dark bleached primary leaves of barley (Kräutler *et al.*, 1991) respectively. Since then considerable progress has been done on the study of the enzymatic degradation of chlorophylls in our (Gossauer, 1994) and other research laboratories (Mühlecker *et al.*, 1993; Hörtensteiner *et al.*, 1995). From the presently available results, a consistent (although in some details still hypothetical) catabolic pathway may be outlined (fig. 1), which comprises all the products of chloro-

⁽¹⁾ Paper presented at the International Workshop "Tetrapyrrole photoreceptors", Freising (Germany), June 5-10, 1995. For Part 7 see Curty *et al.* (1995 a).

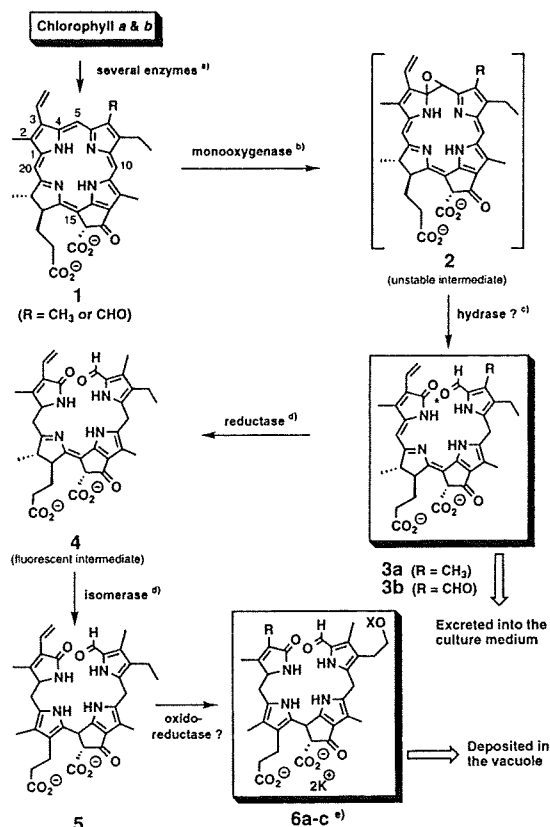


Figure 1. Suggested pathway of the enzymatic degradation of chlorophylls. The probable relationship between the catabolites excreted into the culture medium by bleaching *C. protothecoides* cells and those isolated from dark-bleached primary leaves of barley and from senescent rape cotyledons is shown. The structures of the compounds which have been definitively recognised hitherto as chlorophyll catabolites are framed. (a) presumable successive action of chlorophyllase, magnesium dechelate and hydrolase (Brown *et al.*, 1991). (b) substantiated by ¹⁸O-labelling (Curty *et al.*, 1995). (c) in formula 3 the oxygen atoms proceeding from water and molecular oxygen (*O) are differentiated by an asterisk. (d) suggested by *in vitro* chemical transformation (this work). (e) 6a: R = 1,2 dihydroxyethyl, X = H, as 13² methyl ester in barley (Kräutler *et al.*, 1991); 6b: R = vinyl, X = malonyl K⁺ salt, in rape (Mühlecker *et al.*, 1993); 6c: R = vinyl, X = 1-glucosidyl, in rape (Kräutler, 1995).

phyll breakdown isolated until now from both microalgae and phanerogams, as well as the probable structure of a common unstable intermediate 2 which is formed by the action of a monooxygenase on *O*(13⁴)-demethylpheophorbide (1). As substantiated by *in vivo* ¹⁸O-labelling experiments which have been carried out recently in our laboratory (Curty *et al.*, 1995a), hydrolytic cleavage of the epoxide ring of 2, takes place regioselectively, as expected from the experimentally evidenced chemical reactivity of α -aminoepoxides

(Adam *et al.*, 1992), yielding, without change of the oxidation level, a 19-formyl-1[21H,22H]bilinone 3, which has now been proved to be the primary product of enzymatic cleavage of the chlorophyll macrocycle in *C. protothecoides* (*vide infra*).

RESULTS

Isolation of the primary chlorophyll catabolites of *C. protothecoides*

In our previous work, a mixture of pigments was isolated from the culture medium of *C. protothecoides* after centrifugation of the cell mass, acidification of the supernatant, and extraction of the aqueous phase with ether (fig. 2), a procedure which is substantially the same as used previously by Oshio and Hase (1969) in their study of the bleaching of *Chlorella* cells which takes place when this algae is grown in a medium rich in glucose but poor in nitrogen sources. After chromatographic separation of the above mixture, at least five different formylbilinones derived from Chl-*a* (Engel *et al.*, 1991) and Chl-*b* (Iturraspe *et al.*, 1994) could be purified and characterized by spectroscopic analysis. Furthermore, the structure of the main Chl-*a* degradation product 7a was confirmed both by X-ray diffraction analysis (Engel *et al.*, 1993) and partial synthesis from PPhe-*a* (Iturraspe and Gossauer, 1992). On the other hand, the *in vitro* transformation of pigment 7a into its E-isomer 8a (fig. 3), which had been isolated previously from the culture medium (Iturraspe *et al.*, 1993), suggested that the latter is not a primary product of enzymatic degradation of Chl-*a*.

Most recently, the procedure used to isolate the pigments present in the culture medium of bleaching *C. protothecoides* cells has been modified in that the broth is simply filtered through a membrane filter (*cf.* Methods) and the solid residue is chromatographed on silica gel after esterification with EtOH in the presence of BOP and Et₃N (fig. 2). By this procedure only two pigments were obtained, namely the 19-formyl-1[21H,22H]bilinone derivatives 9a and 9b originating from Chl-*a* and Chl-*b*, respectively. Due to the proneness of chlorophyll derivatives to isomerise at C(13²) (Hynninen, 1991), both 9a and 9b, are isolated as a 3:1 mixture of epimers, as revealed by their ¹H-NMR spectra (*cf.* Methods). Most likely, however, the original absolute configuration at C(13²) is the same in both catabolites as in Chl-*a* and Chl-*b*, respectively.

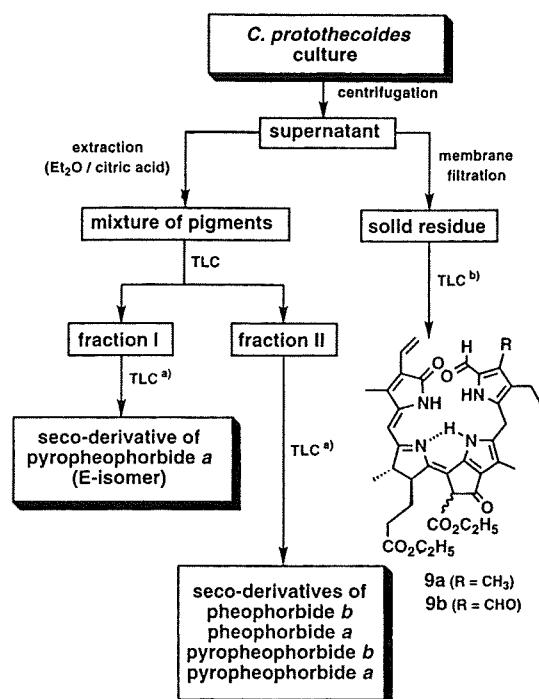


Figure 2. Schematic representation of the isolation procedures used for the separation of the mixture of pigments contained in the culture medium of bleached *C. protothecoides* cells. (a) after esterification with methanol. (b) after esterification with ethanol.

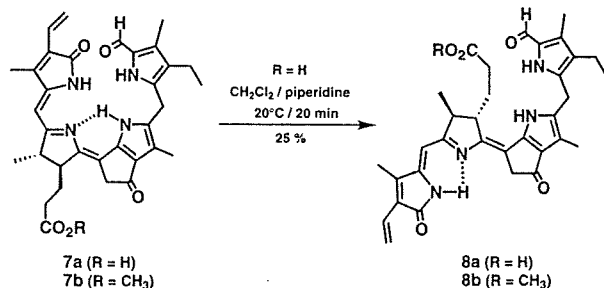


Figure 3. Base-catalysed isomerisation of the Z-isomer 7a into the E-isomer 8a.

As both derivatives 9a and 9b are isolated as their ethyl esters, it must be concluded that the pigments originally present in the culture medium are the corresponding catabolites of *O*(13⁴)-demethylpheophorbide a and b (3a and 3b, respectively) which have to be regarded, therefore, as the primary products of oxidative breakdown of chlorophyll in *C. protothecoides*. Accordingly, the Chl-a catabolite isolated from senescent rape cotyledons has been characterized as the potassium salt of a *O*(13⁴)-

demethylpheophorbide a derivative by Mühlecker *et al.* (1993).

Formylbibinone derivatives related to PPhe-a and PPhe-b as well as to Phe-a and Phe-b, which had been isolated previously by extraction of the culture medium (fig. 2), are probably products of ulterior transformation of 3a and 3b. Actually, both 3a and 3b are β -oxocarboxylic acids, the decarboxylation of which may readily occur at room temperature upon acidification of the culture medium (Jencks, 1969) or during the purification procedure on silica gel plates. On the other hand, small quantities of 3a and 3b, which may persist after the purification, are transformed during the esterification step preceding the chromatographic separation into the corresponding methyl esters (fig. 2), which were considered earlier (Iturraspe *et al.*, 1994) to be the primary products of degradation of Phe-a and Phe-b, respectively.

Partial synthesis of the skeleton of chlorophyll a catabolites from phanerogams

In the course of our systematic analysis of the pigments present in the culture medium of bleaching *C. protothecoides* cells a yellow pigment was isolated, to which, after esterification with methanol, structure 10 was assigned on the basis of its spectroscopic data. From the fact that the pigment, which contains only one asymmetric C-atom, is optically active, an enzymatic transformation of 7a, as the substrate, might be inferred. It has been found, however, that the dimethylester of the latter (7b), as well as its E-isomer 8b, can be transformed irreversibly into 10 in acidic solution (fig. 4), whereby the remarkable high enantiomeric purity of the isomerization product of 7b (up to $98 \pm 2\%$, as determined by ¹H-NMR spectroscopy in the presence of R(-)-ATFE (Pirkle *et al.*, 1977)) does not depend on the bulk of

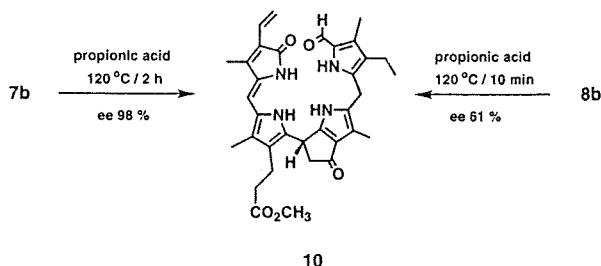


Figure 4. Acid-catalysed rearrangement of both geometric isomers 7b and 8b into the yellow pigment 10. The absolute configuration of the latter is suggested to be R on the basis of mechanistical arguments (Curtis *et al.*, 1995b).

the organic acid used. A detailed study of the mechanism of this transformation will be reported shortly elsewhere (Curty *et al.*, 1995 *b*). From this study the absolute configuration of **10** at C(15) is predicted to be R, irrespective of the geometry (Z or E) of the C(15) = C(16) bond of the substrate. Until now, however, no experimental evidence for this assignment is available.

From the preparative point of view, the transformation of **7b** into **10** provides a straightforward access to the chromophore of the Chl-*a* catabolites isolated in other research groups from dark-bleached primary leaves of barley (Kräutler *et al.*, 1991) and senescent rape cotyledons (Mühlecker *et al.*, 1993).

Actually, catalytic hydrogenation of **7b** yields a fluorescent intermediate **11**, as a mixture of four diastereomers, in 65% yield (*cf.* Methods). Upon treatment with acetic acid at 118°C, **11** is transformed into **12** (fig. 5), which is obtained as a 56 to 44 mixture of two optical active diastereomers with an ee of $45 \pm 2\%$ and $44 \pm 2\%$, respectively.

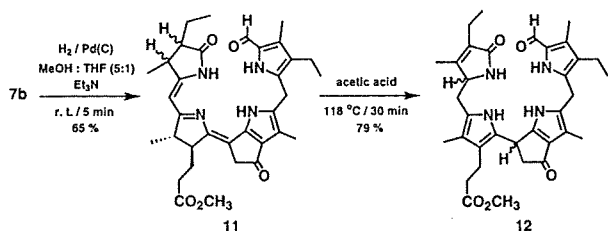


Figure 5. *In vitro* transformation of the main red pigment present after acidification in the culture medium of *C. protothecoides* into **12**. The skeleton of the latter is the same as that of chlorophyll *a* catabolites isolated from phanerogams.

Whereas the migration of the exocyclic C(20) = C(1) bond of **11** into the lactame ring corresponds to the well documented acid-catalysed rearrangement of bilirhodines into urobilines (Köst *et al.*, 1975), the transformation of the pyrroline moiety of **11** into a pyrrole ring may be rationalised in the same way as the isomerization of **7b** to **10**, as the result of two subsequent prototropic shifts. In the porphyrin series, a similar reaction was observed, but not correctly interpreted mechanistically, by Fischer and Spielberger (1935) in the course of their synthesis of pheoporphyrin *a*₅ from Phe-*a*.

DISCUSSION

The chemical transformation of **7b** into **12** strongly suggests a close relationship between the catabolic

pathways of chlorophyll in *C. protothecoides* and in phanerogams. As chlorophytes are generally accepted to be the phylogenetic ancestors of higher plants (van den Hoek *et al.*, 1993), this relationship is not unlikely. Accordingly, the enzymatic transformation of the final product of chlorophyll breakdown in *Chlorella* into the skeleton of the catabolites isolated from both barley and rape leaves would involve a reduction step and two prototropic shifts (fig. 1). However, in contrast to the chemical transformation outlined above, the enzymatic reduction step may be the regioselective hydrogenation of the C(20) = C(1) bond of the substrate rather than hydrogenation of the endocyclic double bond at the lactam ring followed by isomerization. Chemically, a selective hydrogenation of the C(20) = C(1) bond should be, in principle, possible (Gossauer and Blacha-Puller, 1981); in **7b** however, either the presence of a vinyl group conjugated with the C(2) = C(3) bond or the substitution of a pyrroline subunit for an adjacent pyrrole ring renders apparently the endocyclic bond more reactive, so that a regioselective hydrogenation of the C(20) = C(1) bond proved to be non feasible.

Following to the hydrogenation of the C(20) = C(1) bond, the enzyme-catalyzed rearrangement of the pyrroline moiety of **4** into a pyrrole ring may be induced by protonation of the molecule, as in the case of the *in vitro* transformation of **7b** into **10**. The final product **5** differs from the catabolites isolated from barley and rape leaves **6a-c** in the lack of hydroxy groups on the side chains (fig. 1). Whether enzymatic hydroxylation takes place before or after oxidative cleavage of the chlorin macrocycle is for the present not known. As *C. protothecoides* excretes the products of chlorophyll catabolism in the culture medium whereas the phanerogams, which have been investigated so far, accumulated them in vacuoles (Matile, 1995), it may be speculated that the purpose of the enzymatic hydroxylation of the ethyl side chains of the catabolic pigments is to increase their solubility in water. Accordingly, the catabolites isolated from rape cotyledons are either esterified with malonic acid (Mühlecker *et al.*, 1993) or they have been transformed into a glycoside (Kräutler, 1995).

The catabolic pathway depicted on figure 1 has to be considered as a working hypothesis, which needs to be corroborated in the near future by more *in vitro* experiments and, ultimately, by the isolation of the different enzymes involved in the catabolism of chlorophylls. Nevertheless, our present knowledge of this process suggests a consistent picture, in which the

evidence gained from taxonomically different living organisms is, until now, in significant good agreement.

METHODS

Chemicals and materials. All commercially available chemicals were reagent grade; solvents were dried and distilled prior to use. TLC aluminium foils precoated with silica gel 60 PF₂₅₄ (0.2 mm) and preparative TLC plates coated with silica gel 60 PF₂₅₄₊₃₆₆ (1.25 mm thick, 20 × 20 cm) were purchased from Merck (D-6100 Darmstadt, Germany). Ultrafiltration was performed at 1 bar nitrogen pressure in a stirred cell, type 8400, equipped with a Diaflo, PM 10 disc membrane from Amicon, (MA 01915, USA), submerged in an ice bath. (R(-)-ATFE) from Fluka (CH-9474 Buchs, Switzerland) was used as chiral auxiliary for the determination of enantiomeric purity by ¹H-NMR spectroscopy.

Devices. ¹H-NMR were recorded on a Bruker-AM-360 (360.14 MHz) furnished with a data system Aspect 3000. Chemical shifts (δ) are given in ppm downfield from tetramethylsilane, as internal standard, and coupling constants (J) in Hertz. Spin multiplicities are indicated by symbols s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br (broad). Enantiomeric purities were determined by integrating appropriate signals after addition of a tenfold excess (w/w) of R(-)-ATFE to the ¹H-NMR sample. ¹³C-NMR spectra were measured in a Varian Gemini-200 instrument at 50.3 MHz in CDCl₃ as internal standard (δ = 77.0 ppm). ¹H- and ¹³C-NMR assignments are based on NOE correlations and Attached Proton test (APT) and/or chemical shifts. Mass spectra (m/z , relative peak intensity in parenthesis) were obtained with a Vakuum Generator Micromass 7070 E instrument equipped with a DS 11-250 data system from VG Micromass Ltd. (Manchester, UK) using the FAB ionization technique in the positive mode with Xe as primary atom beam at 7 keV and 1 μ A. Samples were first dissolved in MeOH and then added to the 3-nitrobenzyl alcohol matrix. UV/VIS spectra were recorded on a Hewlett Packard-8452 A diode-array spectrophotometer; λ_{\max} are given in nm and relative band intensities in parenthesis; sh = shoulder. CD spectra were obtained with an Jobin-Yvon Auto Dichograph Mark V, $\Delta\epsilon$ (at λ_{\max}) in l mole⁻¹ cm⁻¹ were measured at 25°C.

Isolation of the catabolites. Axenic cells of *Chlorella protothecoides* labelled as ACC no. 25 were grown and bleached as previously described (Engel *et al.*, 1991; Curty *et al.*, 1995a). The final 1 l cell suspension was centrifuged in 200 ml portions at an average of 2500 × g followed by filtration of the supernatant. The pigments contained in the combined, ice-cooled orange-red solutions were separated by ultrafiltration. After about 700 ml of a colorless filtrate has

passed, which takes about 5 h, the adsorptive capacity of the filter-membrane was exhausted and the penetrating pigments start to colour the filtrate. At this point, the filtration process was interrupted and the whole membrane was dried *in vacuo*. Thereafter, the pigments were dissolved in 20 ml absolute EtOH and the solution was directly used for the subsequent esterification.

Esterification and purification of the catabolites. To 20 ml of the above alcoholic pigment solution CH₂Cl₂ (20 ml), Et₃N (2 drops), and BOP (10 mg, 2.26 μ mol) were added, and the mixture was shaken at r.t. under Ar, for 10 h in darkness. Addition of diethyl ether (100 ml) and successive washing of the organic phase with 5% (w/w) aqueous citric acid solution (2 × 40 ml) and water (2 × 40 ml) yields, after drying of the organic layer with Na₂SO₄, filtration and evaporation, a residue which was submitted to prep. TLC, using CH₂Cl₂/ethyl acetate/MeOH (v/v/v, 70/29/1) as eluant. The two separated components were extracted from silica gel with acetone and the eluant evaporated to dryness. Finally, the residues were redissolved in 2 ml of CH₂Cl₂, the solutions were filtered through a cotton-plug and the products obtained after removal of the solvent *in vacuo* were characterized by spectroscopic analysis. *O*-(13⁺)-demethyl-10,22-dihydro-4,5-dioxo-4,5-seco-21H,23H-pheophorbide *a* diethyl ester (**9a**). TLC: R_f = 0.62 (CH₂Cl₂/ethyl acetate/MeOH, v/v/v, 70/29/1), red fluorescent spot. UV/VIS (CH₂Cl₂): 280 (0.70), 312 (1.0), 374 sh (0.36), 428 sh (0.23), 464 sh (0.29), 494 (0.35), 530 (0.32), 582 sh (0.17). ¹H-NMR (CDCl₃): 0.96 (t, J = 7.6, H₃C(8²)), 1.19 (d, J = 7.4, (H₃C(18¹))), 1.22 (t, J = 7.2, propionic ester O-CH₂-CH₃), 1.28 (dd, J = 7.2, 7.2, CO₂CH₂-CH₃), 1.67-1.83, 1.88-2.07 (2 × m, H₂C(17¹)), 2.16 (s, H₃C(2¹)), 2.20-2.29 (m, H₂C(17²)), 2.24 (s, H₃C(7¹)), 2.27 (s, H₃C(12¹)), 2.38 (q, J = 7.6, H₂C(8¹)), 2.46-2.59 (m, HC(17)), 2.72 (br q, J = 7.4, HC(18)), 3.96 (s, H₂C(10)), 4.09 (q, J = 7.1, propionic ester O-CH₂-CH₃), 4.23 (qm, J = 7.2, CO₂-CH₂-CH₃), 4.41 (s, HC(13²)), 5.62 (dd, J = 11.4, 1.7, H_{cis}C(3²)), 5.69 (s, HC(20)), 6.46 (dd, J = 17.6, 1.7, H_{trans}C(3²)), 6.60 (dd, J = 17.6, 11.4, HC(3¹)), 8.54-8.73 (1H) and 9.94-10.21 (2H) (3 × br s, HN), 9.44 (0.25H) and 9.46 (0.75H) (2 × s, HCO) (two signals due to the presence of a 3:1 mixture of epimers). FAB-MS: 532 (42, [M - C₃H₁₀NO]⁺), 669 (100, [M + H]⁺), 691 (41, [M + Na]⁺).

Proton-catalysed rearrangements. 3-Deethyl-10,15,22,24-tetrahydro-4,5-dioxo-3-vinyl-4,5-seco-21H,23H-phytylporphyrin methyl ester (**10**). A solution of **7b** (10 mg, 17 μ mol) in propionic acid (6 ml) was heated under Ar at 118-120°C, in darkness, for about 2 h. After cooling to r.t., the reaction mixture was diluted with CH₂Cl₂ (40 ml) and shaken successively with aqueous 5% NaHCO₃ (2 × 30 ml) and water (2 × 20 ml). The organic phase was dried (Na₂SO₄), filtered and eventually concentrated *in vacuo*. The obtained residue was purified by preparative

TLC using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (v/v, 9/1) as eluant. The product was extracted from the silica gel with acetone and the residue obtained after evaporation of the solvent was redissolved in CH_2Cl_2 (2 ml) and filtered through a cotton-plug to remove the suspended silica gel. After evaporation of the solvent, the residue was dried *in vacuo* for several hours, yielding 82%, **10**, ee $98 \pm 2\%$. TLC: $R_f = 0.75$ ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, v/v, 9/1), yellow non fluorescent spot. UV/VIS (CH_2Cl_2): 278 (0.35), 324 (0.64), 336 sh (0.55), 424 (1.0). CD (CH_2Cl_2): $\Delta\epsilon_{345} = 35.1$, $\Delta\epsilon_{319} = -21.1$. $^1\text{H-NMR}$ ($\text{DMSO}-d_6$): 0.84 (dd, $J = 7.5$ and 7.4 , $\text{H}_3\text{C}(8^2)$), 2.04 (s, $\text{H}_3\text{C}(7^1)$), 2.05 (s, $\text{H}_3\text{C}(2^1)$), 2.15 (s, $\text{H}_3\text{C}(18^1)$), 2.16 (s, $\text{H}_3\text{C}(12^1)$), 2.20-2.35 (m, $\text{H}_2\text{C}(8^1)$ and $\text{H}_2\text{C}(17^1)$), 2.44-2.61 (m, $\text{H}_2\text{C}(17^2)$), 2.69 (dd, $J = 17.4$, 3.1 , $\text{HC}(13^2)$), 3.11 (dd, $J = 17.4$, 7.1 , $\text{HC}(13^2)$), 3.54 (s, CO_2CH_3), 3.74 and 3.82 ($2 \times d$, $J = 15.9$, $\text{H}_2\text{C}(10)$), 4.60 (dd, $J = 7.1$, 3.1 , $\text{HC}(15)$), 5.31 (dd, $J = 11.6$, 2.8 , $\text{H}_{cis}\text{C}(3^2)$), 6.06 (s, $\text{HC}(20)$), 6.20 (dd, $J = 17.7$, 2.8 , $\text{H}_{trans}\text{C}(3^2)$), 6.57 (dd, $J = 17.7$, 11.6 , $\text{HC}(3^1)$), 9.43 (s, HCO), 10.10, 10.24, 11.04 and 11.23 ($4 \times s$, HN). $^{13}\text{C-NMR}$ (CDCl_3): 9.2 (1C) and 9.9 (3C) ($\text{C}(2^1)$, $\text{C}(7^1)$, $\text{C}(12^1)$ and $\text{C}(18^1)$), 15.8 ($\text{C}(8^2)$), 17.3 ($\text{C}(8^1)$), 19.9 ($\text{C}(17^1)$), 22.0 ($\text{C}(10)$), 32.7 ($\text{C}(15)$), 35.4 ($\text{C}(17^2)$), 50.9 ($\text{C}(13^2)$), 51.9 ($\text{C}(\text{OCH}_3)$), 102.0 ($\text{C}(20)$), 118.2 ($\text{C}(3^2)$), 126.1 ($\text{C}(3^1)$), 110.4, 121.8, 122.8, 123.9, 124.0, 126.4, 127.6 (2C), 128.1, 132.8, 133.1, 135.0, 138.2, 141.5, 158.2 and 171.8 ($15 \times \text{quat. C}$ and lactam CO), 173.3 (COO), 176.2 (CHO), 195.0 (CO), FAB-MS: 446 (21, $[\text{M} - \text{C}_8\text{H}_{10}\text{NO}]^+$), 583 (100, $[\text{M} + \text{H}]^+$), 605 (10, $[\text{M} + \text{Na}]^+$).

1,10,15,20,22,24-Hexahydro-4,5-dioxo-4,5-seco-21H,23H-phytoporphyrin methyl ester (**12**): a solution of **11** (9.6 mg, $16.4 \mu\text{mol}$) in acetic acid (4 ml) was heated under Ar at $118-120^\circ\text{C}$ for 30 min. The product, which was isolated as described for **10**, consists of a mixture of two optical active diastereomers **12a** (4.2 mg; 44%) and **12b** (3.4 mg; 35%), which were separated by prep. TLC using acetone/hexane (v/v, 6/4), as eluant.

12a (ee $45 \pm 2\%$): TLC: $R_f = 0.49$ (acetone/hexane, v/v, 1/1), colourless non fluorescent spot. UV/VIS (CH_2Cl_2): 272 (0.66), 318 (1.0). $^1\text{H-NMR}$ ($\text{CDCl}_3 + 2\% \text{CD}_3\text{OD}$): 0.86 (dd, $J = 7.6$, 7.6 , $\text{H}_3\text{C}(3^2)$), 1.08 (dd, $J = 7.6$, 7.6 , $\text{H}_3\text{C}(8^2)$), 1.87 (s, $\text{H}_3\text{C}(18^1)$), 1.93 (s, $\text{H}_3\text{C}(2^1)$), 2.02-2.16 (m, $\text{H}_2\text{C}(3^1)$), 2.16 (s, $\text{H}_3\text{C}(12^1)$), 2.24 (s, $\text{H}_3\text{C}(7^1)$), 2.35-2.43 (m, $\text{H}_2\text{C}(17^1)$), 2.43-2.52 (m, $\text{H}_2\text{C}(8^1)$), 2.61-2.77 (m, $\text{HC}(20)$, $\text{HC}(13^2)$ and $\text{H}_2\text{C}(17^2)$), 2.83-2.89 (m, $\text{HC}(20)$), 3.28 (dd, $J = 17.6$, 6.9 , $\text{HC}(13^2)$), 3.65 (s, CO_2CH_3), 3.87 and 3.97 ($2 \times d$, $J = 16.8$, $\text{H}_2\text{C}(10)$), 3.97-4.06 (m, $\text{HC}(1)$), 4.52 (dd, $J = 7.0$, 3.1 , $\text{HC}(15)$), 9.17 (s, HCO). $^{13}\text{C-NMR}$ ($\text{CDCl}_3 + 2\% \text{CD}_3\text{OD}$): 8.7, 9.0 and 9.3 ($\text{C}(7^1)$, $\text{C}(12^1)$ and $\text{C}(18^1)$), 12.0 ($\text{C}(2^1)$), 12.9 and 15.0 ($\text{C}(3^2)$ and $\text{C}(8^2)$), 16.2 and 16.8 ($\text{C}(3^1)$ and $\text{C}(8^1)$), 19.8 ($\text{C}(17^1)$), 22.5 ($\text{C}(10)$), 27.8 ($\text{C}(20)$), 31.5 ($\text{C}(15)$, 35.3 ($\text{C}(17^2)$), 51.0 ($\text{C}(13^2)$), 51.5 (OCH_3), 60.0 ($\text{C}(1)$), 111.5, 114.9, 117.1, 121.0, 124.6, 124.8, 127.5, 127.9, 131.5, 133.9 (2C), 137.5,

153.1, 157.9 and 175.3 ($14 \times \text{quat. C}$ and lactam CO), 173.9 (COO), 176.1 (CHO), 196.6 (CO). FAB-MS: 448 (11, $[\text{M} - \text{C}_8\text{H}_{12}\text{NO}]^+$), 587 (38, $[\text{M} + \text{H}]^+$), 609 (11, $[\text{M} + \text{Na}]^+$).

12b (ee $44 \pm 2\%$): TLC: $R_f = 0.28$ (acetone/hexane, v/v, 1/1), colourless non fluorescent spot. UV/VIS (CH_2Cl_2): 270 (0.66), 314 (1.0). $^1\text{H-NMR}$ ($\text{CDCl}_3 + 2\% \text{CD}_3\text{OD}$): 0.98 (dd, $J = 7.7$, 7.4 , $\text{H}_3\text{C}(3^2)$), 1.03 (dd, $J = 7.7$, 7.7 , $\text{H}_3\text{C}(8^2)$), 1.94 (s, $\text{H}_3\text{C}(18^1)$), 1.96 (s, $\text{H}_3\text{C}(2^1)$), 2.08-2.30 (m, $\text{H}_2\text{C}(3^1)$ and $\text{HC}(20)$), 2.18 (s, $\text{H}_3\text{C}(12^1)$), 2.22 (s, $\text{H}_3\text{C}(7^1)$), 2.33-2.54 (m, $\text{H}_2\text{C}(8^1)$ and $\text{H}_2\text{C}(17^1)$), 2.61-2.79 (m, $\text{HC}(13^2)$ and $\text{H}_2\text{C}(17^2)$), 3.00 (dd, $J = 14.6$, 3.7 , $\text{HC}(20)$), 3.25 (dd, $J = 17.7$, 6.9 , $\text{HC}(13^2)$), 3.63 (s, CO_2CH_3), 3.80-3.96 (m, $\text{HC}(1)$), 3.87 (s, $\text{H}_2\text{C}(10)$), 4.51 (dd, $J = 6.9$, 2.8 , $\text{HC}(15)$), 9.02-9.32 (br, HCO). $^{13}\text{C-NMR}$ ($\text{CDCl}_3 + 2\% \text{CD}_3\text{OD}$): 8.6 and 9.0 (2C) ($\text{C}(7^1)$, $\text{C}(12^1)$ and $\text{C}(18^1)$), 11.8 ($\text{C}(2^1)$), 12.9 and 14.8 ($\text{C}(3^2)$ and $\text{C}(8^2)$), 16.3 and 16.7 ($\text{C}(3^1)$ and $\text{C}(8^1)$), 19.8 ($\text{C}(17^1)$), 22.6 ($\text{C}(10)$), 29.0 ($\text{C}(20)$), 31.2 ($\text{C}(15)$), 35.5 ($\text{C}(17^2)$), 50.9 ($\text{C}(13^2)$), 51.5 ($\text{C}(\text{OCH}_3)$), 60.8 ($\text{C}(1)$), 111.5, 113.9, 117.5, 122.7, 124.7, 125.2, 127.0, 127.7, 131.1, 133.7 (2C), 136.7, 152.3, 159.0 and 174.8 ($14 \times \text{quat. C}$ and lactam CO), 174.1 (COO), 176.2 (CHO), 197.0 (CO). FAB-MS: 448 (10, $[\text{M} - \text{C}_8\text{H}_{12}\text{NO}]^+$), 587 (32, $[\text{M} + \text{H}]^+$), 609 (14, $[\text{M} + \text{Na}]^+$).

Catalytic hydrogenation. 2,3,3',3'',10,22-Hexahydro-4,5-dioxo-4,5-seco-21H,23H-pyropheophorbide *a* methyl ester (**11**). A solution of **7b** (4.9 mg, $8.41 \mu\text{mol}$), in MeOH/THF (6 ml, v/v, 5/1) containing Et_3N (five drops) and 10% Pd/C-catalyst (3 mg) was stirred in an atmosphere of hydrogen under normal pressure at r.t.. Hydrogenation was interrupted when complete disappearance of the educt was ascertained by TLC analysis of the reaction mixture (about 5 min). Then, the mixture was filtered over a sintered glass disc (*G4*) and the filtrate acidified with acetic acid (3 ml) before it was diluted with CH_2Cl_2 (20 ml). The organic layer was washed with water (3×10 ml), and dried over Na_2SO_4 . After filtration and evaporation of the solvent *in vacuo* the obtained residue was subjected to preparative TLC (acetone/hexane, v/v, 6/4). The product was extracted from silica gel and processed as described for **10**, to yield 3.2 mg (65%) of **11**, as a 1:2:6:12 mixture of four diastereomers, which were transformed into **12** as described above, without previous separation. TLC: $R_f = 0.61$ (acetone/hexane, v/v, 1/1), yellow fluorescent spot. UV/VIS (CH_2Cl_2): 256 (0.71), 306 (1.0), 396 sh (0.45), 416 (0.50), 440 sh (0.41), 472 sh (0.18). $^1\text{H-NMR}$ (CDCl_3): 0.93 (t, $J = 7.3$, $\text{H}_3\text{C}(8^2)$), 1.11 (dd, $J = 7.3$, 7.3 , $\text{H}_3\text{C}(3^2)$), 1.18 (d, $J = 7.3$, $\text{H}_3\text{C}(18^1)$), 1.28 (d, $J = 7.3$, $\text{H}_3\text{C}(2^1)$), 1.62-1.81 (m, $\text{HC}(3^1)$ and $\text{HC}(17^1)$), 1.84-2.07 (m, $\text{HC}(3^1)$ and $\text{HC}(17^1)$), 2.23 (s, $\text{H}_3\text{C}(7^1)$), 2.27 (s, $\text{H}_3\text{C}(12^1)$), 2.21-2.50 (m, $\text{H}_2\text{C}(17^2)$), 2.37 (q, $J = 7.5$, $\text{H}_2\text{C}(8^1)$), 2.52-2.61 (m, $\text{HC}(17)$), 2.61-2.71 (m, $\text{HC}(18)$), 2.72-2.85 (m, $\text{HC}(3)$), 3.27 (d, $J = 19.8$, $\text{HC}(13^2)$), 3.26-3.38 (m, $\text{HC}(2)$), 3.45 (d, $J = 19.8$, $\text{HC}(13^2)$), 3.65 (s, CO_2CH_3), 3.89 and 3.95 ($2 \times d$, $J = 15.9$, $\text{H}_2\text{C}(10)$), 5.22 (d, $J = 3.1$, $\text{HC}(20)$), 8.39,

8.44, 8.47 and 8.61 (4 × br s, HN(23)) (signal pattern due to diastereomeric mixture), 9.40 (0.05H), 9.46 (0.29H), 9.47 (0.58H) and 9.48 (0.08H) (4 × s, HCO) (signal pattern due to diastereomeric mixture), 9.66, 10.07, 10.11 and 10.19 (4 × br s, HN(22)) (signal pattern due to diastereomeric mixture), 10.79-12.52 (br, HN(21)). ¹³C-NMR (CDCl₃): 8.8 and 9.3 (C(7¹) and C(12¹)), 12.4 (C(2¹)), 2 × 14.9 (C(3²) and C(8²)), 17.0 (C(8¹)), 19.0 (C(18¹)), 23.0 (C(10)), 28.2 and 28.3 (C(3¹) and C(17¹)), 30.9 (C(17²)), 37.5 (C(3)), 44.0 (C(13²)), 46.0 (C(2)), 46.7 and 49.5 (C(17) and C(18)), 51.7 (C(OCH₃)), 91.6 (C(20)), 111.2, 112.5, 124.9, 128.7, 131.4, 133.4, 133.8, 133.9, 149.0, 153.9, 159.9, 178.7 and 181.1 (12 × quat. C and lactam CO), 173.2 (C(COO)), 176.5 (CHO), 192.8 (CO). FAB-MS: 450 (51, [M⁺-C₈H₁₀NO]⁺), 586 (100, M⁺), 609 (16, [M + Na]⁺).

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Evidence for a monooxygenase-catalyzed primary process in the catabolism of chlorophyll

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Abstract Only recently have products of the enzymatic breakdown of the chlorophylls been characterized for the first time. All catabolites isolated until now from a chlorophyte and from angiosperms have in common the position at which the primary oxygenative ring cleavage occurs, yielding linear tetrapyrroles (19-formyl-1[21H,22H]bilinones). In vivo studies of $^{18}\text{O}_2$ incorporation in one of the chlorophyll catabolites isolated from *Chlorella protothecoides* show unequivocally that of the two oxygen atoms inserted into the pigment, only the formyl oxygen originates from dioxygen whereas the other one, the lactamic oxygen atom, derives from water. These findings suggest a monooxygenase-catalyzed primary process in the catabolism of chlorophyll.

Key words: Chlorophyll catabolism; Monooxygenase; ^{18}O labeling; *Chlorella protothecoides*; 19-Formyl-1[21H,22H]bilinone

1. Introduction

In contrast to the biosynthesis of the chlorophylls, which has been elucidated in great detail in the past decades, virtually nothing was known until recently about the chemical structures and the enzymes involved in the degradation of these essential pigments of life [1]. The global chlorophyll turnover which is estimated at about 10^9 t annually is particularly conspicuous in its spectacular degradation during autumnal senescence of leaves.

During the last four years two different research groups have succeeded independently from each other in the structure elucidation of different catabolites of the chlorophylls isolated from such diverse phyla as the chlorophyte *Chlorella protothecoides* [2,3], and from angiosperms like the monocot *Hordeum vulgare* vc. Gerbel (barley, [4]), and the dicot *Brassica napus* L. (rape, [5]). It was surprising to notice that both in algae and higher plants the chromophore of the photosynthetic pigments is regioselectively cleaved by oxygenation at the C4-C5 bond of the chlorophyll macrocycle yielding bile-pigment-like products which resemble the parent chlorophylls by the presence of the characteristic condensed cyclopentane ring (cf. 5 in Fig. 1). In contrast to heme catabolism in which the *meso* carbon atom C5 is released as carbon monoxide during ring cleavage, chlorophyll catabolites retain this atom as a formyl group attached to ring B. It has been anticipated that oxygenolysis of the macrocycle may occur by the action of a dioxygenase, which recognizes a dephytylated form of chlorophyll, presumably pheophorbide [6]. The elucidation of the mechanism by which

this unusual enzymatic oxidative ring opening occurs is the objective of the present investigation.

2. Materials and methods

2.1. Chemicals

All commercially available chemicals were reagent grade; solvents were distilled prior to use. Thin layer chromatography (TLC) aluminium foils precoated with silica gel 60 F₂₅₄ (0.2 mm) and preparative TLC plates coated with silica gel 60 PF₂₅₄₊₃₆₆ (1.25 mm thick, 20 × 20 cm) were purchased from E. Merck (D-6100 Darmstadt, Germany). Oxygen mixtures were prepared from natural oxygen taken from an ordinary gas cylinder and from isotopic oxygen, consisting of a mixture of 98.505 atom% ^{18}O , 0.063 atom% ^{17}O , and 1.432 atom% ^{16}O , contained in a break seal flask purchased from Cambridge Isotope Laboratories Inc., Andover, MA 01810-5413, USA.

2.2. Mass spectrometry

Mass spectra were obtained with a Vacuum Generator Micromass 70 70E instrument equipped with a DS 11-250 data system from VG Micromass Ltd. (Manchester, UK) using the fast atom bombardement (FAB) ionization technique in the positive mode with Xe as primary atom beam at 7 keV and 1 μA . Samples were first dissolved in MeOH and then added to the glycerol/1-thioglycerol (1:1) matrix.

2.3. In vivo incubation procedure

In each experiment, 100 ml cultures of *Chlorella protothecoides* (5×10^7 cells/ml) have been grown heterotrophically on 5% glucose in darkness as previously described [3]; air was displaced by nitrogen in an air-tight shaking vessel containing the algae culture. The device was immediately connected through a silicon tubing to a graduated reservoir containing ^{18}O -enriched oxygen maintained under atmospheric pressure. Thus, one run was carried out with $^{16,18}\text{O}_2$: $^{18,18}\text{O}_2$ = 55.5:44.5 and another with $^{16,16}\text{O}_2$: $^{18,18}\text{O}_2$ = 11:89. At the beginning of the experiment, an incubation atmosphere of about 80% nitrogen and 20% oxygen was established by suction of oxygen from the reservoir, draining off a calculated amount of aqueous 4 N NaOH solution contained in a compartment of the reactor. Continuous oxygen flow into the reactor was driven by absorption of the carbon dioxide, liberated during glucose consumption, in the NaOH solution mentioned above. Bleaching was interrupted after 48 h during which 250 ml oxygen was consumed. The main chlorophyll *a* catabolite 5 was isolated, purified and analyzed by mass spectroscopy as described before [2,7].

3. Results

In a previous work [7] we demonstrated that photooxygenative ring opening of the chlorophyll *a* derivative, pyropheophorbide *a* methyl ester Cd(II), in non-aqueous solutions occurs by the so-called 'one molecule mechanism', i.e. both terminal oxygen atoms derive from the same oxygen molecule suggesting that singlet oxygen is involved in the formation of a dioxetane derivative as intermediate. The resulting product resembles the natural chlorophyll catabolites.

In order to gain an insight into the reaction mechanism of the primary enzymatic reaction in chlorophyll catabolism, the origin of the terminal oxygen atoms present in the isolated products is of predominant interest. As a matter of fact, the

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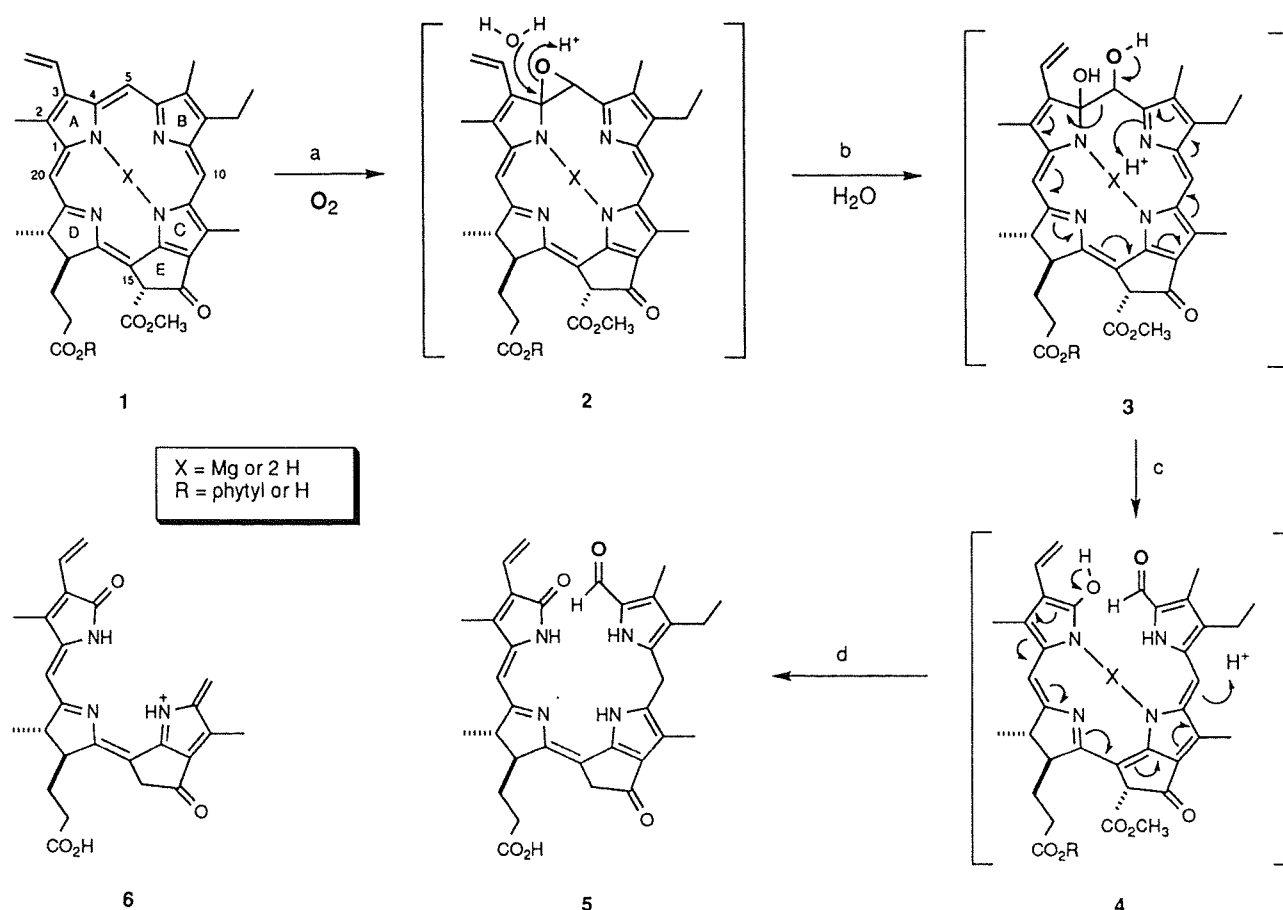


Fig. 1. Mechanism proposed for the ring cleavage of chlorophyll *a* involving the action of a monooxygenase in the key step. The following sequence for the ring opening is suggested: (a) addition of a monooxygenase-activated oxygen molecule to the C4–C5 double-bond yields the oxirane derivative 2; (b) regioselective cleavage of the oxirane ring by spontaneous or enzymatic (epoxide hydrolase) hydrolysis results in the formation of intermediate 3, in which the oxygen label remains bound at position C5; (c) a *retro*-aldol condensation type reaction affords 4; (d) prototropic rearrangement of the latter and subsequent decarbomethoxylation on C13² eventually yields the isolated chlorophyll *a* catabolite 5. Included in the figure is the structure of the mass spectroscopic fragment ion (6) at *m/z* 432, in which the lack of ¹⁸O label supports the proposed mechanism.

green algae *C. protothecoides* offers essential advantages compared with pluricellular organisms for *in vivo* studies, namely because: (i) the structures of the catabolites isolated from angiosperms are more complex in so far as they contain oxygen atoms on peripheral substituents in addition to the two oxygen atoms concerned with the ring cleavage reaction; and (ii) handling of submerge cultures simplifies the design of the incubation device.

The methodology followed in the present work parallels that which was developed originally by Brown et al. [8] to elucidate the mechanism of heme degradation *in vivo*. Thus, in order to differentiate between the four conceivable reaction mechanisms (Table 1) batches of living green cells of *C. protothecoides* were incubated with specific isotopic mixtures of ^{18,18}O₂ and ^{16,16}O₂ during the process of heterotrophic growth (bleaching) in which the chlorophylls *a* and *b* are catabolized. The isotopic composition of the main chlorophyll *a* catabolite excreted into the culture medium during the process of bleaching was analyzed, after chromatographic separation, by FAB mass spectroscopy as previously described [7].

Although under these conditions the main catabolite is difficult to separate from the accompanying pheophorbide *a* analogue as well as from the corresponding chlorophyll *b* cat-

abolites [3], the mixture of the four pigments was directly used for FAB mass spectrometric analysis, since it contains more than 90% of the chlorophyll *a* catabolite 5, which has the lowest molecular mass of all components. Actually, the closest contaminant is at least +14 mass units apart from the center of the molecular ion, and as such does not interfere with the mass spectroscopic measurements. FAB mass spectra obtained from the incubation samples were statistically analyzed and are juxtaposed in Fig. 2. The resulting data have been mathematically transformed into the relative amount of pigment labeled with none, one or two ¹⁸O atoms (Table 1), as formerly developed [7].

The comparison of the experimentally observed labeling pattern of the pigment with the predicted incorporation rates matches a 'monohydrolytic cleavage mechanism'. Nevertheless, this assignment could be delusive taking into account the possibility of a hydrolytic exchange of one of the terminal oxygen atoms in pigment 5 after a ring cleavage which may take place by a dioxygenation mechanism involving either one or two oxygen molecules.

It was found, however, that both ¹⁸O atoms remain in a double labeled pigment, which was synthesized by photooxygenation (*vide supra*), when the latter was incubated in the bleach-

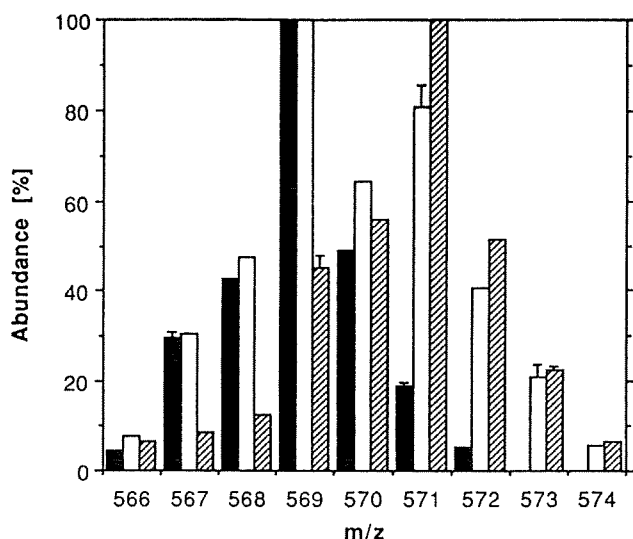


Fig. 2. Juxtaposed intensity patterns in the range of the molecular ion peaks of the FAB spectra of **5**, isolated from the culture media of *Chlorella protothecoides* using: natural O_2 (black bars); $^{16,16}O_2:^{18,18}O_2 = 55.5:44.5$ (white bars); and $^{16,16}O_2:^{18,18}O_2 = 11:89$ (hatched bars). Each intensity value represents the mean of ten repeated-scan FAB mass spectra, standard deviations of relevant signals are indicated on the top of the bars.

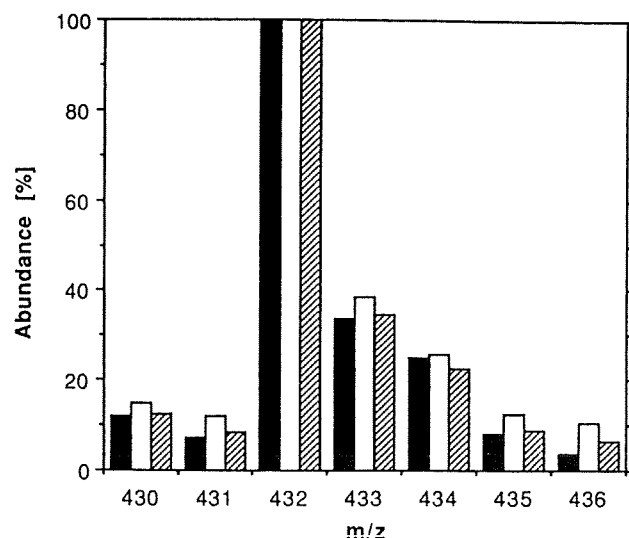


Fig. 3. Juxtaposed intensity patterns in the range of the peaks associated with the fragment ion **6** in the FAB spectra of **5**, isolated from the culture media of *Chlorella protothecoides*, using: natural O_2 (black bars); $^{16,16}O_2:^{18,18}O_2 = 55.5:44.5$ (white bars); and $^{16,16}O_2:^{18,18}O_2 = 11:89$ (hatched bars).

ing medium at pH 6.8. In aqueous KOH or HCl solutions, on the contrary, only one oxygen atom is readily exchanged. Mass spectroscopic investigation of the product after hydrolysis shows that the oxygen label remains in fragment ion **6** (actually the basis peak), carrying the lactam group [7]. Therefore, hydrolytic exchange of the oxygen atom must have occurred, as expected, at the formyl group and not at the lactam group.

On the contrary, mass spectrometric analysis of the products obtained from incubation experiments *in vivo* reveals the lack of label in the fragment ion **6** in all samples investigated, thus proving that the ^{18}O atom must be located in the formyl group (Fig. 3). The exclusive detection of ^{18}O in the formyl group of **5** rules out the possibility of a dioxygenation mechanism involving one or two oxygen molecules with subsequent hydrolytic label exchange and suggests a primary process in the catabolism of chlorophyll which is catalyzed by a monooxygenase.

4. Discussion

The reaction sequence outlined in Fig. 1 is suggested on a chemical plausible basis. Both epoxide formation and hydrolytic epoxide ring cleavage are well-documented enzymatic processes [9]. As epoxide **2** and the final catabolite **5** are on the same oxidation level, addition of water is sufficient for the transformation of the former into the latter. This is in contrast to the related oxidative ring opening of heme by heme oxygenase, in which water is not involved in the scission step.

Actually, the specific substrate of the monooxygenase is still unknown. However, as pheophytin *a* is less prone to oxidation than chlorophyll *a* [10], the latter may be favored as a substrate for the enzyme. It also remains unclear at what point in the catabolic process the hydrolysis of the phytol ester takes place. On the generally accepted assumption that chlorophytes are the phylogenetic ancestors of higher plants [11], the chlorophyll

Table 1

Calculated statistical incorporation of ^{18}O into formylbilinone **5** as predicted for the four possible cleavage mechanisms compared with the experimentally observed data

Reaction mechanism	$^{16,16}O_2:^{18,18}O_2 = 55.5:44.5$			$^{16,16}O_2:^{18,18}O_2 = 11:89$		
	m/z	m/z	m/z	m/z	m/z	m/z
One oxygen molecule	569	571	573	569	571	573
Two oxygen molecules	55.5	0	44.5	11	0	89
Mono hydrolytic	30.8	49.4	19.8	1.2	19.6	72.2
Double hydrolytic	55.5	44.5	0	11	89	0
	100	0	0	100	0	0
Experimentally observed	53.3 ± 2.2	40.8 ± 5.3	5.9 ± 3.2	14.9 ± 4.1	81.4 ± 2.1	3.7 ± 1.1

In the 'double hydrolytic mechanism' both terminal oxygen atoms in the pigment are derived from water, whereas in the 'mono-hydrolytic mechanism' one atom proceeds from dioxygen and the other from water. 'One oxygen molecule mechanism' means that both atoms are derived from a single dioxygen molecule, whereas in the 'two oxygen molecules mechanism' they originate from two different dioxygen molecules [8]. Thus, the values account for the relative amounts of each molecular species in the sample containing none ($m/z = 569$), one ($m/z = 571$) and two ($m/z = 573$) ^{18}O atoms. In the calculation, small amounts of the mixed dioxygen species $^{18,16}O_2$ present in the gas mixtures have been neglected for simplicity, the resulting error is within the experimental accuracy. Experimentally observed data were calculated from the values shown in Fig. 2 according to our previous work [7] and are presented as mean ± S.E.M.

cleavage mechanism suggested in this work should be common to all photosynthetic eukaryotes.

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MECHANISM OF PHOTOOXYGENATION OF THE Cd (II) COMPLEX OF PYROPHEOPHORBIDE *a* METHYL ESTER*

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Abstract—Photooxygenation of (pyropheophorbido *a* methyl ester)cadmium (II) was studied using $^{18,18}\text{O}_2$ labeling of the molecular oxygen required for cleavage of the macrocycle. After reductive demetallation of the primary oxidation product (4,5-dioxo-4,5-secopyropheophorbido *a* methyl ester)cadmium (II), the isotope content of formylbilinone **4a** was analyzed by repeated-scan fast atom bombardment mass spectrometry. Comparison of the spectroscopic data of the labeled pigment **4a** with the statistical probabilities of ^{18}O isotope incorporation calculated for four possible reaction mechanisms clearly proves that photooxidative ring cleavage occurred by the one-molecule mechanism, *i.e.* the terminal oxygen atoms of **4a** were derived from one oxygen molecule. Furthermore, a study of the exchange of the ^{18}O -labeled atoms revealed that no exchange occurs within the pH 4.5–9.5 range. In stronger alkaline or acidic solutions, only the oxygen atom of the formyl group is exchanged. Hydrolysis of the methyl ester group of **4a** was achieved, without loss of the ^{18}O label on the formyl group, at pH 7.2 in the presence of pig liver esterase.

INTRODUCTION

The discovery that the zinc chelate of pyropheophorbide *a* methyl ester (PPheM)‡ can be photooxidized *in vitro* yielding bilindione derivatives² stimulated a series of experiments on the photooxidation of other metal complexes,³ which culminated with a partial synthesis of the main product of enzymatic degradation of chlorophyll *a* (Chl) in *Chlorella protothecoides* cells from (pyropheophorbido *a* methyl ester)cadmium (II) (**1**).⁴ Thus, this unprecedented synthesis of an intermediate on the pathway of chlorophyll catabolism satisfies from a formal point of view the definition of a “biomimetic” transformation,⁵ although it is moot whether this denomination is also valid when the mechanisms of the reactions involved in the process are taken into consideration. Therefore, in order to gain an insight into the mechanism of the photooxidation of pyropheophorbide *a* (PPhe) *in vitro*, experiments were carried out using $^{18,18}\text{O}_2$ -enriched oxygen. The results of these investigations are reported in the present work. An account of related experiments, which were carried out *in vivo*, using *C. protothecoides* cells, will be given shortly (N. Engel, C. Curty and A. Gossauer, in preparation).

The methodology followed in the present work parallels that which was developed originally by Brown and coworkers^{6–9} to elucidate the mechanism of oxidative ring cleavage of heme *in vivo* and applied later in other laboratories for similar investigations of the chemical and photochemical oxygena-

tion of tetrapyrrolic pigments.^{10–12} Thus, mixtures of $^{18,18}\text{O}_2$ and $^{16,16}\text{O}_2$ were used in all experiments in order to differentiate between the four conceivable reaction mechanisms, in which the two oxygen atoms of the final reaction product may originate from O_2 and/or H_2O . The isotope content of the product was analyzed, as commonly, by mass spectrometry, a method that provides enough accuracy in order to determine the number of ^{18}O atoms incorporated in the molecule. However, the plurality of natural isotopes of cadmium renders a direct analysis of the primary product **3**, obtained on photooxygenation of **1**, uncertain, and for this reason, the metal ion was removed by ligand reduction with NaBH_4 (*cf.* Iturraspe and Gossauer⁴), prior to mass spectrometric analysis. Optimal peak intensities in the range of the molecular ion of both **4b**, which is identical with the main Chl catabolite isolated from *C. protothecoides*, and its corresponding methyl ester **4a**, were obtained using the fast atom bombardment (FAB) ionization method. As matrix, a mixture of glycerol and 1-thioglycerol proved to be superior to either glycerol alone or 3-nitrobenzyl alcohol.

MATERIALS AND METHODS

Chemicals. All commercially available chemicals were reagent grade; solvents were distilled prior to use. Pig liver esterase lyophilized powder (220 U/mg) was a gift from Fluka Chemie AG, (CH-9470 Buchs). Isotopically enriched oxygen was purchased in a break seal flask from Cambridge Isotope Laboratories Inc. (Andover, MA 01810-5413, USA), containing 98.5 atom% ^{18}O . Thin-layer chromatography (TLC) aluminum foils precoated with silica gel 60 (0.2 mm) and preparative TLC plates precoated with silica gel 60 PF₂₅₄₊₃₆₆ (1.25 mm thick, 20 × 20 cm) were purchased from E. Merck (D-6100 Darmstadt, Germany).

Devices. Fast atom bombardment mass spectrometry was performed in the positive mode with a Vacuum Generator Micromass 70 70E instrument equipped with an 11-250 data system from VG Micromass Ltd. (Manchester, UK). The instrument was equipped with a standard VG FAB source and an Ion Tech gun operating with

*Part 6 in the series “Chlorophyll catabolism.” For part 5 see Iturraspe *et al.*¹

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‡Abbreviations: Chl, chlorophyll *a*; DMSO, dimethyl sulfoxide; EtOH, ethanol; FAB, fast atom bombardment; MeOH, methanol; PPhe, pyropheophorbide *a*; PPheM, pyropheophorbide *a* methyl ester; TLC, thin-layer chromatography.

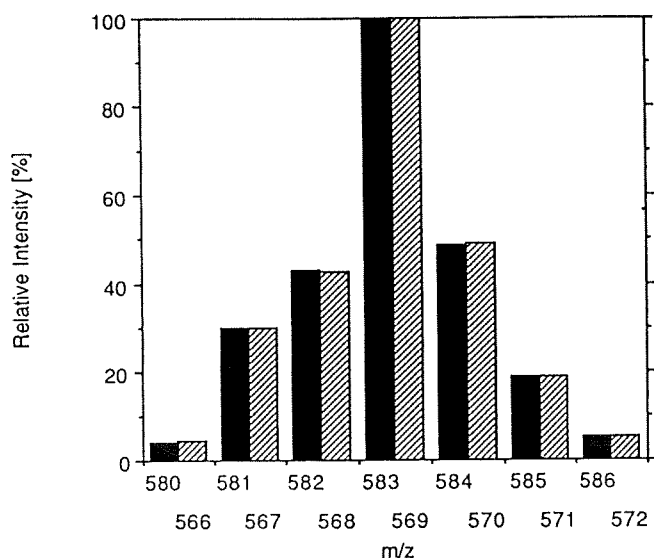


Figure 1. Juxtaposed intensity patterns of the FAB spectra of the unlabeled pigments: 4a (■) and 4b (▨).

Xe as primary atom beam at 7 keV and 1 μ A. Samples were first dissolved in methanol (MeOH) and then added to glycerol/1-thioglycerol (1:1). All quantitative mass measurements are averages of at least 10 independent spectra.

Determination of the isotopic composition from mass spectroscopic data. Owing to the complex peak pattern intrinsic to the FAB method, a simple algebraic approach has been developed for the calculation of the isotopic content of O atoms in the samples analyzed. Figure 1 displays the juxtaposed mass spectra of unlabeled 4a and 4b in the range of the molecular ion peak $[M+H]^+$, which is flanked by some peaks of higher and lower m/z ($[M+H\pm n]^+$).

Algebraic equations have been developed under the assumption that the mass spectral pattern displayed by the unlabeled pigment would be identical when labeled with either one or two ^{18}O -atoms but shifted upward by two or four mass units, respectively. Therefore, only the two peaks, which are ± 2 mass units away from the main signal, will overlap with the neighboring peaks. The calculating procedure is illustrated in Fig. 2. The relations x and y of the intensities of the satellite signals to the intensity of the main peak have been determined in the FAB spectrum of the unlabeled compounds and found to be $x = 0.2972$ and $y = 0.1892$. In order to obtain the intensity of each signal due to the content of a particular isotopic species, the contributions xb , yb , xc and ya must be subtracted from the intensity of the principal peaks A, B and C. Thus, the segments a , b and c represent the intensities of each isotopically labeled species. They can be determined from the three obvious equations: $A = a + xb$, $C = c + yb$ and $B = b + xc + ya$ (cf. Fig. 2). Insertion of the two former equations into the latter and transformation to $b = (B - Ay - Cx)/(1 - 2xy)$ allows calculation of the unknown fractions from the peak intensities and finally the percentage of pigment labeled with none, one or two ^{18}O atoms.

In accordance with Brown and coworkers,⁶⁻⁹ the statistical probabilities of the incorporation of the ^{18}O isotope in the terminal carbonyl groups of the pigment have been calculated for different ratios of isotopic dioxygen under consideration of four conceivable reaction mechanisms (cf. Table 1). Hydrolytic mechanisms could be excluded because the photooxygenations were performed in nonaqueous CH_2Cl_2 solutions. Nevertheless, the labeling pattern for the mono- and double hydrolytic mechanisms were included, because they are indispensable for the evaluation of the hydrolysis products 4b.

Labeling experiments. Photooxygenation of 1 was performed under similar conditions as described previously.⁴ For the sake of simplicity, the reaction was performed in a closed reactor, refrigerated in an ice bath, in an atmosphere containing a mixture of $^{18,18}\text{O}_2$ (97%), and natural $^{16,16}\text{O}_2$ (99.6%) from a cylinder. Thus, one run was carried out with $^{18,18}\text{O}_2$ (45 mL) and $^{16,16}\text{O}_2$ (20 mL), which

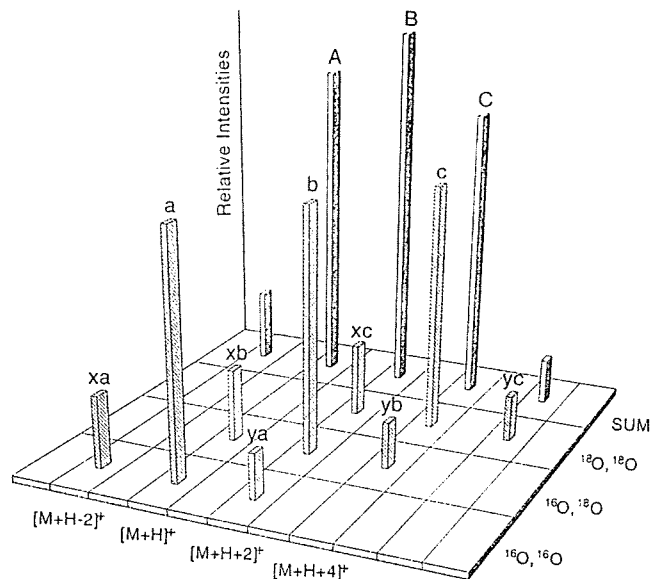


Figure 2. Constructed FAB spectrum of an analytical sample containing equal amounts of molecules with none, one and two ^{18}O atoms. The figure shows the overlapping peaks in the region of the molecular ion and the parameters from which algebraic equations have been derived. A, B and C indicate the total ion current at m/z $[M+H]^+$, $[M+H+2]^+$ and $[M+H+4]^+$, respectively. The contribution to the peak intensities due to the content of O isotopes in the sample is represented by the segments a , b and c . The quotients x and y are obtained from the FAB spectrum of the unlabeled sample by division of the peak intensities at m/z $[M+H-2]^+$ and $[M+H+2]^+$, respectively, by the peak intensity of the molecular ion ($[M+H]^+$).

results in an approximately $^{18,18}\text{O}_2$: $^{16,16}\text{O}_2$ mixture of 69:31. This was taken out of the gas containers with a syringe and injected into the previously evacuated reactor. Another run was carried out with a mixture of $^{18,18}\text{O}_2$ (25 mL) and $^{16,16}\text{O}_2$ (40 mL), which corresponds to a $^{18,18}\text{O}_2$: $^{16,16}\text{O}_2$ mixture of approximately 38:62.

The final reduction and demetallation step to 4a was accomplished

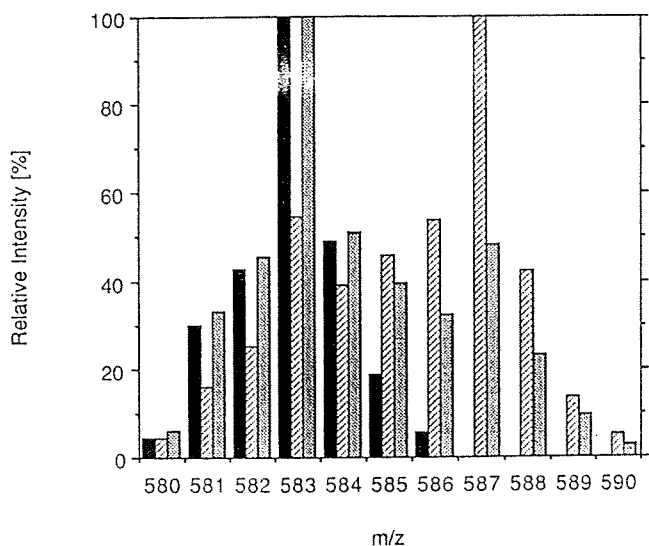


Figure 3. Juxtaposed intensity patterns in the range of the molecular ion peaks in the FAB spectra of 4a, obtained by photooxygenation of 1 and subsequent reductive demetallation, using: natural O_2 (■), $^{18,18}\text{O}_2$ / $^{16,16}\text{O}_2$ (69:31; vol/vol) (▨) and $^{18,18}\text{O}_2$ / $^{16,16}\text{O}_2$ (38:62; vol/vol) (▤).

Table 1. Calculated statistical incorporation of ^{18}O into formylbilinones **4a** and **4b** as predicted for the four possible mechanisms of photooxygenation of **1***

Reaction mechanism	$^{18,18}\text{O}_2/^{16,16}\text{O}_2 = 69:31$			$^{18,18}\text{O}_2/^{16,16}\text{O}_2 = 38:62$		
	$^{16}\text{O}, ^{16}\text{O}$	$^{18}\text{O}, ^{16}\text{O}$	$^{18}\text{O}, ^{18}\text{O}$	$^{16}\text{O}, ^{16}\text{O}$	$^{18}\text{O}, ^{16}\text{O}$	$^{18}\text{O}, ^{18}\text{O}$
One oxygen molecule	31	0	69	62	0	38
Two oxygen molecules	9.6	42.8	47.6	38.5	47.1	14.4
Monohydrolytic	31	69	0	62	38	0
Double hydrolytic	100	0	0	100	0	0

*The values account for the relative amounts of each molecular species in the sample containing none, one and two ^{18}O atoms. In the calculations, small amounts of the mixed dioxygen species $^{16,18}\text{O}_2$ present in the gas mixture have been neglected for simplicity. The resulting error is within the experimental accuracy of about ± 6 (mol%) for each species determined from 10 independent mass spectra.

as described earlier.⁴ The isolation procedure was slightly modified in order to avoid label exchange during the work-up procedure. Thus, after dilution with water (200 mL), the reaction mixture was acidified with citric acid (1.0 g) and extracted with diethyl ether (2×50 mL). The combined organic extracts were washed with water (2×30 mL), dried (Na_2SO_4) and evaporated *in vacuo*. The residue was purified by TLC using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (9:1) as eluant. The product was extracted from the silica gel with MeOH and the residue obtained after evaporation of the solvent was redissolved in CH_2Cl_2 (1 mL) containing two drops of ethanol (EtOH) and filtered through a glass frit (G4) to remove the suspended silica gel. After evaporation of the solvent, the residue was dried *in vacuo* for several hours and stored at -18°C under Ar.

Hydrolysis experiments. Enzymatic procedure: A solution of labeled **4a** (3 mg, 5×10^{-3} mmol) in dimethyl sulfoxide (DMSO; 0.5 mL) was added to stirred potassium phosphate buffer (10 mL, 0.1 M, pH 7.2). After addition of esterase from pig liver (5 mg), the solution was shaken for 8 h at 25°C in the darkness. Thereafter, the mixture was diluted with H_2O (90 mL) and the product (2.6 mg, 89%) was isolated and stored as described above.

In basic medium: To a solution of labeled **4b** (1 mg, 1.8×10^{-3} mmol) in MeOH/ H_2O (40 mL, 3:1), KOH (100 mg, 1.8 mmol) was added and the mixture was shaken for 5 h at room temperature in the darkness under Ar. After dilution with H_2O (100 mL), the product (0.73 mg, 73%) was isolated and stored as described above.

In acidic medium: The reaction was carried out under the same conditions as the preceding with labeled **4b** (1 mg) dissolved in MeOH/ H_2O (40 mL, 3:1), to which 37% aqueous HCl (100 μL , 1 mmol) was added, yielding 0.82 mg (82%) of the product.

RESULTS AND DISCUSSION

Table 1 summarizes the relative intensities of the peaks in the region of the molecular ion of formylbilinone **4a** and **4b**, predicted for two different ratios of $^{18,18}\text{O}_2$ to $^{16,16}\text{O}_2$, as a function of four possible photooxygenation mechanisms. The corresponding experimental values are shown in Table 2.

Comparison of the data shows unequivocally that both oxygen atoms present in pigment **4a** belong to the same isotope. Minor amounts of species, in which only one of the two atoms is an ^{18}O isotope, are within the range of experimental error. Thus, the oxidative ring cleavage of **1** proceeds by the so-called one oxygen molecule mechanism, *i.e.* both oxygen atoms incorporated in the photooxidation product originate from the same dioxygen molecule. Studies on the photooxygenation of metallo-*meso*-tetraphe-nylporphins¹¹ and bacteriopheophorbide *c*,¹³ the macrocycle of which is cleaved at the methene bridge bound to a methyl group led to the same conclusion. To the best of our knowledge, however, the mechanism of photooxygenation of a chlorin derivative, the macrocycle of which is cleaved at an unsubstituted *meso* position, had not yet been investigated before.

Most likely, singlet dioxygen, the formation of which is probably photosensitized by the substrate of the reaction itself (**1**), is involved as the reactive species. Regioselective [$\pi 2 + \pi 2$] cycloaddition of the latter to the C(4)–C(5) bond of **1** yields presumably a dioxetane **2**, which decomposes spontaneously, in the usual way, to yield the formylbilinone Cd (II) complex **3** (*cf.* Scheme 1). The same mechanism was assumed, without the evidence of isotope labeling experiments, by Fuhrhop *et al.*¹³ in their study of the photooxygenation of the Zn (II) complex of octaethylchlorin. The fact that in the latter reaction two regioisomeric dioxetanes were formed, whereas in the case of **1** the cleavage of the α -methene bridge is regioselective, suggests a higher degree of bond localization in the phorbine chromophore, which is probably due to the cyclopentane ring condensed to the chlorin macrocycle.

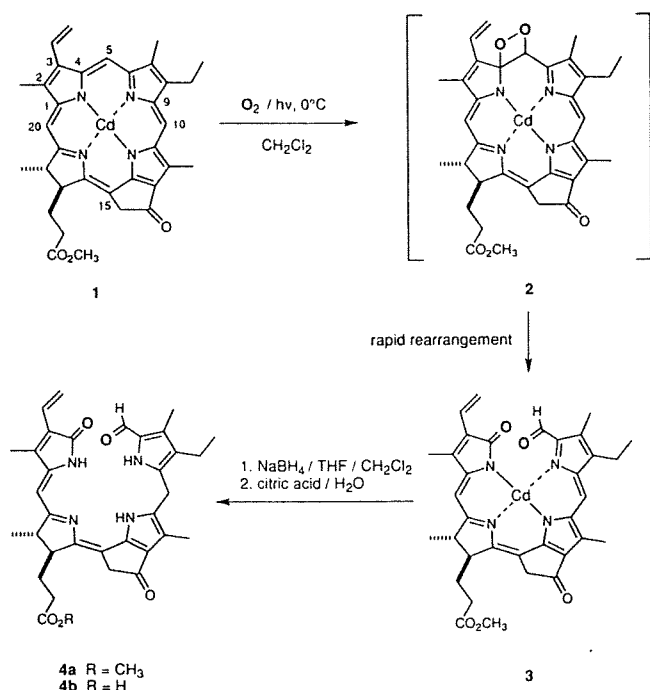
The above results indicate moreover that during reductive

Table 2. Relative contributions to the peak intensities in the region of the molecular ion calculated from the parameters a, b and c (*cf.* Fig. 2) determined experimentally from the FAB mass spectra of formylbilinones **4a** and **4b** (*cf.* Fig. 3 and 4)

Reaction product	$^{18,18}\text{O}_2/^{16,16}\text{O}_2 = 69:31$			$^{18,18}\text{O}_2/^{16,16}\text{O}_2 = 38:62$		
	$[\text{M}+\text{H}]^+$	$[\text{M}+\text{H}+2]^+$	$[\text{M}+\text{H}+4]^+$	$[\text{M}+\text{H}]^+$	$[\text{M}+\text{H}+2]^+$	$[\text{M}+\text{H}+4]^+$
4a	33.3*	4.1	62.6	64.4*	4.8	30.8
4b (enzymatic hydrolysis of 4a)	33.9†	4.1	62.0	—	—	—
4b (basic treatment of 4b)	37.1†	62.9	0	—	—	—
4b (acidic treatment of 4b)	33.3†	66.7	0	—	—	—

* $[\text{M}+\text{H}]^+ = 583$.

† $[\text{M}+\text{H}]^+ = 569$.



Scheme 1. Suggested reaction mechanism of the photooxidative ring cleavage of (pyropheophorbido *a* methyl ester)cadmium (II) (1).

demetallation and isolation of the final product 4a, no significant exchange of the lactam or formyl oxygen atoms occurs. On the contrary, attempts to obtain the carboxylic acid 4b, by hydrolysis of the corresponding ester 4a resulted under both basic and acidic conditions in the rapid exchange of one of the isotopically labeled oxygen atoms (*cf.*, Table 2). In aqueous HCl no hydrolysis of the ester group took place; under basic conditions the formyl ^{18}O atom is exchanged

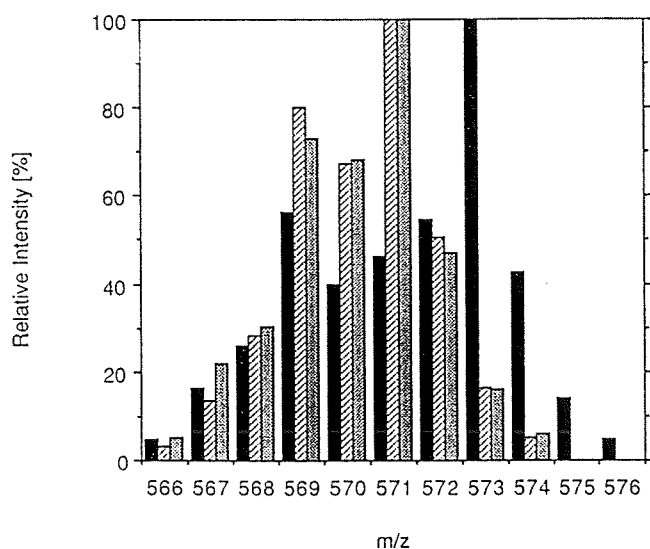


Figure 4. Juxtaposed intensity patterns in the range of the molecular ion peaks in the FAB spectra of 4b, isolated after enzymatic hydrolysis of labeled 4a (■), treatment of labeled 4a with base (▨) and treatment of labeled 4b with acid (▤).

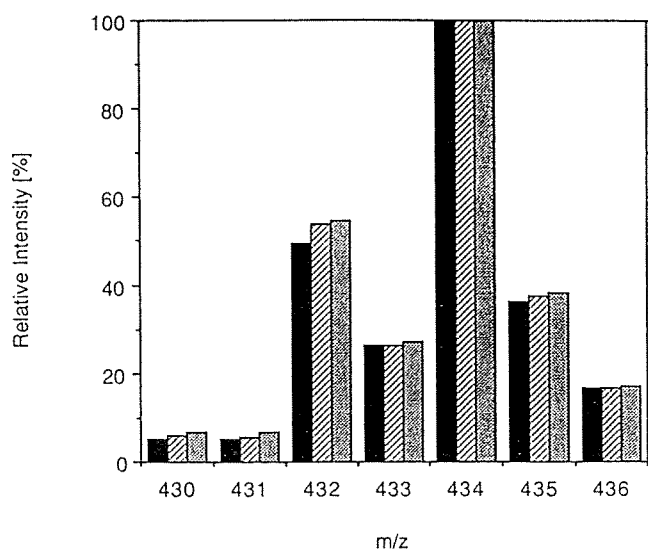
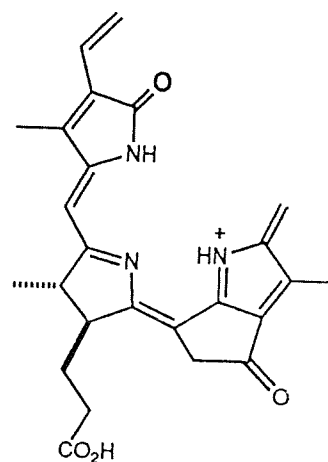


Figure 5. Juxtaposed intensity patterns in the range of the peaks associated with the fragment ion 5 in the FAB spectra of 4b, isolated after enzymatic hydrolysis of labeled 4a (■), treatment of labeled 4b with base (▨) and treatment of labeled 4b with acid (▤).

quantitatively before the hydrolysis of the ester is complete (*vide infra*).

On the other hand, hydrolysis of the methyl ester 4a could be achieved in 89% yield, with entire retention of the ^{18}O labels, in phosphate buffer solution in the presence of pig liver esterase (*cf.* Table 2). Even under controlled hydrolytic conditions (either with 45 mM KOH or 25 mM HCl aqueous solutions) the carboxylic acid 4b lost one of the isotopically labeled oxygen atoms, thus confirming the results obtained on the attempted ester hydrolysis (*cf.* Table 2).

Detailed information concerning the chemoselectivity of the oxygen exchange process could be obtained by virtue of the presence of an m/z 432 peak (actually the base peak) in the FAB mass spectrum of unlabeled 4b, which arises from the molecular ion by fragmentation at the C(9)–C(10) bond.



Thus, it became evident from the comparison of the spectra of labeled and unlabeled **4b** that the incorporated ^{18}O atom remains in the tripyrrolic fragment **5**, because the corresponding m/z 434 peak is shifted by two mass units relative to the unlabeled compound (*cf.* Fig. 5). Therefore, exchange of the oxygen atom occurs, as expected, at the formyl group and not at the lactam group of **4a** and **4b**. On the other hand, no exchange could be observed (even after 4 months at pH 9.5 and room temperature), when the unlabeled pigment **4b** was dissolved in H_2^{18}O containing either KH_2PO_4 (pH 4.5) or K_2HPO_4 (pH 9.5).

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98. Synthesis of Chlorophyll *a* Labeled at C(3²) from Pheophorbide *a* Methyl Ester

by Rolf Fischer, Norbert Engel, Arthur Henseler, and Albert Gossauer*

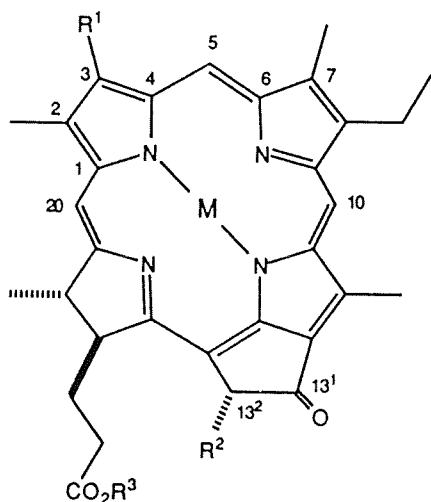
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(28.III.94)

[3²-¹⁴C]Chlorophyll *a* (**10b**) was synthesized from pheophorbide *a* methyl ester (**5a**) in a seven-step partial synthesis. The key intermediate pheophorbide *a* methyl ester (**6**) was obtained by ozonolysis of the vinyl group of **5a** in 91 % yield. Selective reduction of the CHO group of **6** gave the corresponding alcohol **7**, and conversion of the latter to the phosphonium bromide **8** yielded after *Wittig* reaction with [¹⁴C]paraformaldehyde, [3²-¹⁴C]pheophorbide *a* methyl ester (**5b**). The final transformation to the title compound was achieved by acid hydrolysis of **5b**, esterification with natural phytol to [3²-¹⁴C]pheophytin *a* (**9b**), and eventual insertion of a Mg²⁺ ion.

1. Introduction. – Different endogenous chlorophyll *a* (**10a**) catabolites which are excreted by some photoautotrophic microorganisms into the culture medium, under particular growing conditions, were recently isolated and characterized as 19-formyl-1-oxobilin derivatives in our laboratory [1]. However, the fate of these primary products of chlorophyll breakdown in living organisms, which lack the faculty of getting rid of them, is still an enigma. Obviously, the study of the enzymatic degradation of chlorophyll would be facilitated by the availability of derivatives labeled with isotopes which could be used as substrates of both *in vivo* and *in vitro* transformations.

Though isotopic labels, which are not exchangeable under physiological conditions, can be introduced in chlorophyll derivatives by different procedures already reported in the literature, none of them proved to be suitable for our purpose. Thus, the (acidic) H-atoms of the Me(12¹) group can be exchanged by ²H-atoms [2] and ¹⁸O can be incorporated in the CO(13¹) group by enzymatic methods [3]. However, whereas the former



	R ¹	R ²	R ³	M
1a, b^a	CH=CH ₂	H	Me	2 H
2	CHO	H	Me	2 H
3	CH ₂ OH	H	Me	2 H
4	[CH ₂ P(Ph) ₃] ⁺ Br ⁻	H	Me	2 H
5a, b^a	CH=CH ₂	CO ₂ Me	Me	2 H
6	CHO	CO ₂ Me	Me	2 H
7	CH ₂ OH	CO ₂ Me	Me	2 H
8	[CH ₂ P(Ph) ₃] ⁺ Br ⁻	CO ₂ Me	Me	2 H
9a, b^a	CH=CH ₂	CO ₂ Me	phytyl	2 H
10a, b^a	CH=CH ₂	CO ₂ Me	phytyl	Mg

^a) [3²-¹⁴C]-enriched.

procedure is limited to labeling with H-isotopes, the latter is handicapped by the inaccuracy of the analytical methods available for the detection of the ^{18}O -isotope. On the other hand, the most commonly used enzymatic incorporation of 5-aminolaevulinic acid enables certainly to introduce isotopic labels in chlorophyll *a* *in situ* [4], but it is neither appropriated for large-scale preparations nor for specific labeling at predetermined positions [5].

The present work deals with a seven-step high-yield partial synthesis of chlorophyll *a* selectively enriched at C(3²) with the ^{14}C -isotope. Obviously, the method is also applicable to the synthesis of the corresponding ^{13}C -labeled derivative. The starting material is pheophorbide *a* methyl ester (**5a**)¹⁾ which can be conveniently obtained from the commercially available spray-dried cyanobacterium *Spirulina geitlerii* [6] [7].

2. Results and Discussion. – As chlorophyll derivatives bearing a COOMe group at C(13²) are prone to oxidation at this position (so-called allomerization reaction [8a]), the reaction sequence used for the synthesis of isotopically enriched chlorophyll *a* was previously elaborated with pyropheophorbide *a* methyl ester [6] (**1a**)¹⁾, which is considerably less sensitive to O_2 . Thus, following the procedure previously described for the transformation of protoporphyrin IX dimethyl ester into 3,8-diformyldeuteroporphyrin IX dimethyl ester [9], ozonolytic cleavage of the vinyl group of **1a** led to pyropheophorbide *d* methyl ester (**2**) in 92% yield.

Attempts to reconstruct the vinyl group of **1a** by *Wittig* reaction of aldehyde **2** with an excess of triphenylphosphonium methylide according to the procedure described for the synthesis of 7-demethyl-7-vinylchlorophyll *a* from chlorophyll *b* [10] afforded the desired pyropheophorbide *a* methyl ester in only 16% yield, along with several uncharacterized by-products. Under similar conditions, the Zn complex of **2** gave (pyropheophorbidato *a* methyl ester)zinc(II) in 32% yield.

Better results were obtained, however, using the reverse strategy for the formation of the vinyl C=C bond. Thus, reconstruction of the vinyl group of **1a** was achieved in three steps: *i*) reduction of the aldehyde group of **2** with tetrabutylammonium triacetoxyborohydride [11], a reagent which did not affect the carbonyl group at C(13¹), *ii*) transformation of the resulting primary alcohol **3** into the corresponding phosphonium salt **4** by reaction with $\text{PPh}_3/\text{CBr}_4$ [12], and *iii*) *Wittig* reaction of **4** with paraformaldehyde at 90° in the presence of 1,2-epoxypropane as base [13]. The overall yield of the whole reaction cycle amounted to 65%. A similar series of transformations was used by *Wasielewski et al.* [14] for the synthesis of a pyrochlorophyllide *a* dimer.

By the same procedure, the labeled [$3^2\text{-}^{14}\text{C}$]pheophorbide *a* methyl ester (**5b**) was obtained *via* **6–8** from **5a** in 36% overall yield using ^{14}C -enriched paraformaldehyde. Some loss of material was due to de(methoxycarbonylation) of **5b** in the last step of the reaction sequence, which led to **1b** as by-product. Moreover, as during the reaction cycle, epimerization at C(13²) took place to some extent (*cf.* [8b]), the obtained product **5b** consisted of a mixture of both epimers in a ratio of 85:15, which were easily determined by NMR spectroscopy. After crystallization, the (13²*R*)-isomer was usually enriched, depending on the solvent used.

¹⁾ As the first step of the synthesis is carried out in $\text{MeOH}/\text{CHCl}_3$ which contains H_2SO_4 , esterification takes place during the reaction so that the parent carboxylic acid can be used instead of the methyl ester.

Both intermediates **3** and **6** are potential precursors of naturally occurring tetrapyrrolic pigments. Thus, the Ni complex of **3** was isolated, as the free propanoic acid (Tunichlorin), from the Caribbean tunicate *Trididemnum solidum* [15], and aldehyde **6** is the chromophore of chlorophyll *d*, an accessory pigment of some *Rhodophyceae* [16].

After acid-catalyzed hydrolysis of the methyl propanoate group of **5b**, the propanionic acid chain was esterified with natural (7*R*,11*R*,*E*)-phytol in the presence of [(1*H*-benzotriazol-1-yl)oxy]tris(dimethylamino)phosphonium hexafluorophosphate (BOP) [17] to yield [3^2 - ^{14}C]pheophytin *a* (**9b**). The required phytol was obtained by saponification of crude chlorophyll extracts from *Spirulina* according to Willstätter's procedure [18]. Magnesium insertion into **9b** using [2,6-di(*tert*-butyl)-4-methylphenoxy]magnesium iodide [19] afforded [3^2 - ^{14}C]chlorophyll *a* (**10b**) in 61 % overall yield from **5b**.

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Experimental Part

General. All commercially available chemicals were reagent grade, solvents for chemical reactions and chromatography were generally dried and distilled prior use. Spray-dried *Spirulina geitleri* was purchased from Dr. W. Behr, D-53225 Bonn, and [^{14}C]paraformaldehyde (1 mCi, 490 mCi/g) from ICN Radiochemicals. Ozone generator: model 502 from Fischer Labor und Verfahrenstechnik AG. TLC Monitoring: E. Merck silica gel 60 (0.2 mm) precoated aluminium foils. Prep. TLC: silica gel 60 PF₂₅₄+366 plates (1.25 mm thick, 20 × 20 cm) from E. Merck. Scintillation counter: Beckmann LS 1800. M.p.'s: Kofler hot-stage apparatus from Thermovar, C. Reichert AG, Vienna; uncorrected. UV/VIS (CH₂Cl₂): Hewlett-Packard-8452A diode-array spectrophotometer; λ_{max} in nm and ϵ , in l·mol⁻¹·cm⁻¹, in parentheses. IR (KBr): Mattson-5000. FT-IR Spectrometer; $\tilde{\nu}$ in cm⁻¹. ^1H -NMR (CDCl₃): Bruker-AM-360 equipped with a data system Aspect 3000; chemical shifts δ in ppm rel. to SiMe₄ as internal standard, *J* in Hz; assignments, if necessary, confirmed by $^1\text{H}\{^1\text{H}\}$ -NOE difference correlations. FAB-MS: Vacuum Generator Micromass 7070 E equipped with a data system DS 11-250 from VG Micromass Ltd., Manchester, UK; 6 kV acceleration voltage under Xe bombardment and NBA (3-nitrobenzyl alcohol) as matrix; *m/z* (rel. peak intensity).

Pyropheophorbide a Methyl Ester (= Methyl (3*S*,4*S*)-9-Ethenyl-14-ethyl-4,8,13,18-tetramethyl-20-oxophorbine-3-propanoate; **1a**). Air was displaced by Ar from a suspension of phosphonium salt **4** (59.7 mg, 68 μmol) and paraformaldehyde (2.05 mg, 68 μmol) in THF (5 ml), contained in a sealable Pyrex glass tube. Then the tube was immersed in an ultrasonic bath for 5 min, 1,2-epoxypropane (0.1 ml) added, and the mixture heated at 90° for 3 h. After cooling to r.t., the solvent was evaporated and the remaining residue submitted to prep. TLC (hexane/acetone 4:3): 24.3 mg (65%) of **1a** (*R*_f 0.67). ^1H -NMR: identical with reported data [20].

Pyropheophorbide d Methyl Ester (= 3-Devinyl-3-formylpyropheophorbide a Methyl Ester = Methyl (3*S*,4*S*)-14-Ethyl-9-formyl-4,8,13,18-tetramethyl-20-oxophorbine-3-propanoate; **2**). A stirred soln. of **1a** (1.037 g, 1.89 mmol) in CHCl₃/MeOH 1:1 (60 ml) containing conc. H₂SO₄ (2.1 ml) was chilled to -70° before a stream of O₃/O₂ (0.34 mmol/l O₃) was bubbled through a sintered glass disc (*G4*) with a flow rate of 35 l/h. After 13 min, Me₂S (590 μl , 8 mmol) was added and stirring continued in the dark for 12 h at r.t. Thereafter, the mixture was diluted with CHCl₃ (150 ml) and shaken successively with H₂O and 6% aq. HCl soln. (3 × 60 ml; hydrolysis of the dimethyl acetal at C(3¹) formed during ozonolysis). Finally, the org. layer was washed with H₂O, dried (Na₂SO₄), and evaporated and the residue crystallized from CH₂Cl₂/hexane: 957 mg (92%) of **2**. Spectroscopic data: identical with those reported in [21].

3-Devinyl-3-(hydroxymethyl)pyropheophorbide a Methyl Ester (= Methyl (3*S*,4*S*)-14-Ethyl-9-(hydroxymethyl)-4,8,13,18-tetramethyl-20-oxophorbine-3-propanoate; **3**). To a stirred soln. of tetrabutylammonium borohydride (512 mg, 2.0 mmol) in dry CH₂Cl₂ (10 ml), AcOH (0.34 ml, 6.0 mmol) was slowly added under Ar. Stirring was continued for 1 h at r.t. before the mixture was cooled to 4° and added to an ice-cooled soln. of **2** (220 mg, 0.4 mmol) in CH₂Cl₂ (10 ml). After 3 h at 4°, the mixture was poured into 5% (v/v) aq. AcOH (60 ml) and shaken with CHCl₃. The org. layer was washed twice with H₂O, dried (Na₂SO₄), and evaporated. Purification of the residue by prep. TLC (hexane/acetone 4:3) yielded the product (*R*_f 0.39), which was dissolved in CH₂Cl₂,

precipitated by addition of hexane, and finally recrystallized from acetone: 181 mg (82%) of **3**. M.p. 234–235°. Spectroscopic data: in agreement with those reported in [15].

{3-Devinyl-3-[(triphenylphosphonio)methyl]pyropheophorbide a Methyl Ester} Bromide (= {Methyl (3*S*,4*S*)-14-Ethyl-4,8,13,18-tetramethyl-20-oxo-9-[(triphenylphosphonio)methyl]phorbine-3-propanoate} Bromide; **4**). CBr₄ (230 mg, 0.69 mmol) and PPh₃ (217 mg, 0.83 mmol) were added under Ar in the dark to a stirred soln. of **3** (170 mg, 0.308 mmol) in dry THF (40 ml). After 2 d at r.t., anal. TLC (acetone/hexane 1:1) showed complete transformation of **3** to the corresponding bromide (*R_f* 0.54). More PPh₃ (408 mg, 1.23 mmol) was added at once and the mixture refluxed for 4 h. After evaporation the residue was purified by prep. TLC (CH₂Cl₂/MeOH 9:1) to afford the product (*R_f* 0.65) which was recrystallized from CH₂Cl₂/hexane: 256 mg (95%) of **4**. M.p. > 300°. ¹H-NMR (5.2 · 10⁻³ M): 9.32 (*s*, CH(10)); 8.86 (*s*, CH(20)); 8.25 (*s*, CH(5)); 7.79–7.71 (*m*, 6 H_o of Ph); 7.67–7.62 (*m*, 3 H_p of Ph); 7.47–7.41 (*m*, 6 H_m of Ph); 6.81, 6.64 (*ABX*, *J* = 15.3, 14.5, CH₂(3¹)); 5.26, 5.11 (*AB*, *J* = 19.9, CH₂(13²)); 4.36 (*qd*, *J* = 7.3, 2.0, CH(18)); 4.27–4.23 (*m*, CH(17)); 3.65 (*s*, MeO); 3.61 (*s*, Me(12¹)); 3.48 (*q*, *J* = 7.6, CH₂(8¹)); 2.92 (*s*, Me(7¹)); 2.71 (*d*, *J* = 2.7, Me(2¹)); 2.70–2.53 (*m*, CH₂(17¹)); 2.38–2.16 (*m*, CH₂(17²)); 1.72 (*d*, *J* = 7.3, Me(18¹)); 1.56 (*t*, *J* = 7.6, Me(8²)); –1.43, –3.24 (2 br. *s*, 2 NH). FAB-MS: 797 (27, [*M* – Br]⁺), 535 (100).

[3²-¹⁴C]Pheophorbide a Methyl Ester (= Methyl (3*S*,4*S*,21*R*)-9-[2-¹⁴C]Ethenyl-14-ethyl-21-(methoxycarbonyl)-4,8,13,18-tetramethyl-20-oxophorbine-3-propanoate; **5b**). Following the procedure described for **1a**, **5b** (20.6 mg, 50%; 14.7 mCi/mmol) and **1b** (3.3 mg, 9%; 14.7 mCi/mmol) were obtained from the reaction of **8** (64 mg, 68 μmol) with [¹⁴C]paraformaldehyde (2.05 mg; 1 mCi). Both **5b** and **1b** (*R_f* 0.62 and 0.67, resp.) were separated by prep. TLC (hexane/acetone 4:3). ¹H-NMR of **5b**: identical with data given in [7].

Pheophorbide d Methyl Ester (= 3-Devinyl-3-formylpheophorbide a Methyl Ester = Methyl (3*S*,4*S*,21*R*)-14-Ethyl-9-formyl-21-(methoxycarbonyl)-4,8,13,18-tetramethyl-20-oxophorbine-3-propanoate; **6**). A soln. of **5a** (349 mg, 0.575 mmol) in CHCl₃/MeOH 1:1 (30 ml) containing conc. H₂SO₄ (1.05 ml) was ozonolyzed for 8 min as described for **2**. The product was recrystallized from CH₂Cl₂/hexane: 318 mg (91%) of **6**. M.p. > 256° (dec.). UV/VIS (CH₂Cl₂): 696 (56100), 634 (6700), 580 (sh), 552 (12200), 520 (10800), 484 (sh), 426 (70000), 386 (64300), 332 (sh), 310 (22200). IR: 3379w, 2957m, 2927m, 2867m, 1737s, 1700s, 1677s, 1618m. ¹H-NMR (6.5 · 10⁻³ M): 11.54 (*s*, CHO); 10.33 (*s*, CH(5)); 9.65 (*s*, CH(10)); 8.85 (*s*, CH(20)); 6.33 (*s*, CH(13²)); 4.54 (*qd*, *J* = 7.3, 1.8, CH(18)); 4.31–4.27 (*m*, CH(17)); 3.90 (*s*, Me(13⁵O)); 3.78 (*s*, Me(2¹)); 3.77 (*q*, *J* = 7.6, CH₂(8¹)); 3.75 (*s*, Me(12¹)); 3.57 (*s*, Me(17⁵O)); 3.33 (*s*, Me(7¹)); 2.71–2.51 (*m*, CH₂(17¹)); 2.36–2.17 (*m*, CH₂(17²)); 1.85 (*d*, *J* = 7.3, Me(18¹)); 1.73 (*t*, *J* = 7.6, Me(8²)); –0.06, –1.98 (2 br. *s*, 2 NH). FAB-MS: 608 (95, *M*⁺), 609 (100).

3-Devinyl-3-(hydroxymethyl)pheophorbide a Methyl Ester (= Methyl (3*S*,4*S*,21*R*)-14-Ethyl-9-(hydroxymethyl)-21-(methoxycarbonyl)-4,8,13,18-tetramethyl-20-oxophorbine-3-propanoate; **7**). As described for **3**, from **6** (300 mg, 0.49 mmol). Recrystallization from acetone yielded 265 mg (88%) of **7**. M.p. > 235° (dec.). UV/VIS (CH₂Cl₂): 664 (42500), 604 (8300), 556 (sh), 534 (9600), 504 (10200), 470 (sh), 410 (99200), 376 (59000), 332 (21900). IR (KBr): 3452m, 3398w, 2955m, 2925m, 2869m, 1738s, 1679s, 1618s. ¹H-NMR (5.7 · 10⁻³ M): 9.45 (*s*, CH(10)); 9.39 (*s*, CH(5)); 8.54 (*s*, CH(20)); 6.21 (*s*, CH(13²)); 5.85 (*s*, CH₂(3¹)); 4.44 (*qd*, *J* = 7.3, 2.0, CH(18)); 4.20–4.16 (*m*, CH(17)); 3.87 (*s*, Me(13⁵O)); 3.64 (*s*, Me(12¹)); 3.63 (*q*, *J* = 7.6, CH₂(8¹)); 3.58 (*s*, Me(17⁵O)); 3.38 (*s*, Me(2¹)); 3.21 (*s*, Me(7¹)); 2.65–2.48 (*m*, CH₂(17¹)); 2.32–2.16 (*m*, CH₂(17²)); 1.79 (*d*, *J* = 7.3, Me(18¹)); 1.67 (*t*, *J* = 7.6, Me(8²)); 0.32, –1.76 (2 br. *s*, 2 NH). FAB-MS: 610 (100, *M*⁺), 611 (87).

{3-Devinyl-3-[(triphenylphosphonio)methyl]pheophorbide a Methyl Ester} Bromide (= {Methyl (3*S*,4*S*,21*R*)-14-Ethyl-21-(methoxycarbonyl)-4,8,13,18-tetramethyl-20-oxo-9-[(triphenylphosphonio)methyl]phorbine-3-propanoate} Bromide; **8**). As described for **4**, from **7** (250 mg, 0.409 mmol). The product (*R_f* 0.62) was recrystallized from CH₂Cl₂/hexane: 344 mg (90%) of **8**. M.p. > 200° (dec.). UV/VIS (CH₂Cl₂): 674 (46300), 616 (6300), 564 (sh), 536 (7700), 508 (9400), 470 (sh), 412 (79800), 380 (51700), 328 (16600). IR (KBr): 3390w, 3056w, 2955m, 2925m, 2867m, 1736s, 1699s, 1618m. ¹H-NMR (4.3 · 10⁻³ M): 9.32 (*s*, CH(10)); 8.90 (*s*, CH(20)); 8.27 (*s*, CH(5)); 7.79–7.71 (*m*, 6 H_o of Ph); 7.67–7.62 (*m*, 3 H_p of Ph); 7.47–7.41 (*m*, 6 H_m of Ph); 6.81, 6.61 (*ABX*, *J* = 15.3, 14.5, CH₂(3¹)); 6.27 (*s*, CH(13²)); 4.35 (*qd*, *J* = 7.3, 2.0, CH(18)); 4.19–4.15 (*m*, CH(17)); 3.90 (*s*, Me(13⁵O)); 3.62 (*s*, Me(17⁵O)); 3.61 (*s*, Me(12¹)); 3.40 (*q*, *J* = 7.6, CH₂(8¹)); 2.90 (*s*, Me(7¹)); 2.68 (*d*, *J* = 3.0, Me(2¹)); 2.72–2.51 (*m*, CH₂(17¹)); 2.33–2.21 (*m*, CH₂(17²)); 1.73 (*d*, *J* = 7.3, Me(18¹)); 1.53 (*t*, *J* = 7.6, Me(8²)); –0.06, –3.62 (2 br. *s*, 2 NH). FAB-MS: 855 (32, [*M* – Br]⁺), 593 (100).

[3²-¹⁴C]Pheophytin a (= (7*R*,11*R*,*E*)-3,7,11,15-Tetramethylhexadec-2-en-1-yl (3*S*,4*S*,21*R*)-9-[2-¹⁴C]-Ethenyl-14-ethyl-21-(methoxycarbonyl)-4,8,13,18-tetramethyl-20-oxophorbine-3-propanoate; **9b**). A soln. of **5b** (20.6 mg, 34 μmol, 0.54 mCi) in 24% aq. HCl soln. (60 ml) was stirred at r.t. for 90 min. The soln. was then diluted with H₂O (100 ml) and extracted with CH₂Cl₂. The org. phase was washed with H₂O, dried (Na₂SO₄), and evaporated. To the remaining residue in CH₂Cl₂ (5 ml), [(1*H*-benzotriazol-1-yl)oxy]tris(dimethylamino)phosphonium hexafluorophosphate (164 mg, 0.37 mmol), Et₃N (75 mg, 0.74 mmol), and (7*R*,11*R*,*E*)-phytol (100 mg,

0.34 mmol) were added. The mixture was stirred for 24 h at r.t. and the product isolated by prep. TLC (hexane/acetone 4:3): 25.7 mg (87%) of **9b** (R_f 0.69, 14.7 mCi/mmol).

[3²,¹⁴C]Chlorophyll *a* (= {(7*R*,11*R*,*E*)-3,7,11,15-Tetramethylhexadec-2-en-1-yl (3*S*,4*S*,21*R*)-9-[2-¹⁴C]-Ethenyl-14-ethyl-21-(methoxycarbonyl)-4,8,13,18-tetramethyl-20-oxophorbine-3-propanoate(2-)-N²³,N²⁴,N²⁵,N²⁶}magnesium; **10b**). A mixture of **9b** (25.7 mg) and unlabeled pheophytin *a* (**9a**; 25 mg) was converted to **10b** according to [19]: 36.3 mg (70%, 7.5 mCi/mmol) of **10b**.

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CHLOROPHYLL CATABOLISM.* ISOLATION AND STRUCTURE ELUCIDATION OF CHLOROPHYLL *b* CATABOLITES IN *CHLORELLA PROTOTHECOIDES*†

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Key Word Index—*Chlorella protothecoides*; chlorophyll *a*; chlorophyll *b*; catabolism; degradation; 19-formyl-1[21H, 22H] bilinones.

Abstract—When grown in a medium rich in glucose but poor in a nitrogen source, *Chlorella protothecoides* cells excrete different pigments which have been characterized as products of chlorophyll *a* catabolism. Now, degradation products of chlorophyll *b* have been isolated and characterized for the first time.

INTRODUCTION

Not long ago, the structure of a red bilin derivative excreted in the culture medium by *Chlorella protothecoides* when this microalga is grown in a medium rich in glucose but poor in nitrogen [2] has been elucidated in our laboratory [3], and later confirmed both by partial synthesis [4] and X-ray diffraction analysis [1]. This pigment proved to be a product of the enzymatic cleavage of pyropheophorbide *a*, formed by regioselective oxidative scission of the C-4=C-5 bond of the tetrapyrrole macrocycle. As the resulting bilin derivative and its *E*-isomer [5] are the main products in the mixture of pigments present in the culture medium, decarbomethoxylation at C-13² must take place to a large extent either chemically in the culture medium or enzyme-catalysed within the cells prior to excretion. It was moreover surprising that no catabolites of chlorophyll *b* were isolated in the first instance, although the ratio of the latter to chlorophyll *a* in most photoautotrophic organisms is in the range 1:2 to 1:3 [6].

The present work deals with the isolation and structure elucidation of three new bilins from *C. protothecoides* cultures, one derived from chlorophyll *a*, prior to decarbomethoxylation, and two from chlorophyll *b*. The latter are indeed the first products of enzymatic degradation of chlorophyll *b* which have been characterized so far.

RESULTS AND DISCUSSION

On the plausible assumption that no unexpected molecular changes occur on esterification of the pigments excreted into the culture medium by *C. protothecoides* (cf. [5]), their structures **1a**, **2a** and **3a** may be inferred from those of the corresponding methyl esters mentioned above.§ The latter have been elucidated by spectroscopic analysis, particularly homonuclear NOE difference experiments (Tables 1–3).

The most striking feature in the ¹H NMR spectra of both chlorophyll *b* catabolites **2b** and **3b** is the presence of two signals (δ 10.27, 9.86 and 10.28, 9.86 ppm, respectively) in the characteristic range of formyl protons. The observed NOE correlations confirm that cleavage of the tetrapyrrole macrocycle has occurred, as in the bilin previously characterized [3], at the C-4=C-5 bond. According to the NOE experiments (Tables 1–3) pigments **1b** and **2b** have *Z*, *syn* geometry at both exocyclic double bonds. Although in the case of pigment **3b** a clear reciprocal interaction between H-17 or H₂-17¹ and H₂-13² could not be observed, the same geometry has been assigned to the C-15=C-16 bond, on the strength of its absorption and fluorescence spectra which are both similar to those of its previously characterized chlorophyll *a* analogue. On the other hand, the observed NOE connections between H₃-12¹ and H₃-8² in the spectrum of pigment **2b** indicate free rotation of the formylpyrrole moiety on the C-9–C-10 and C-10–C-11 bonds.

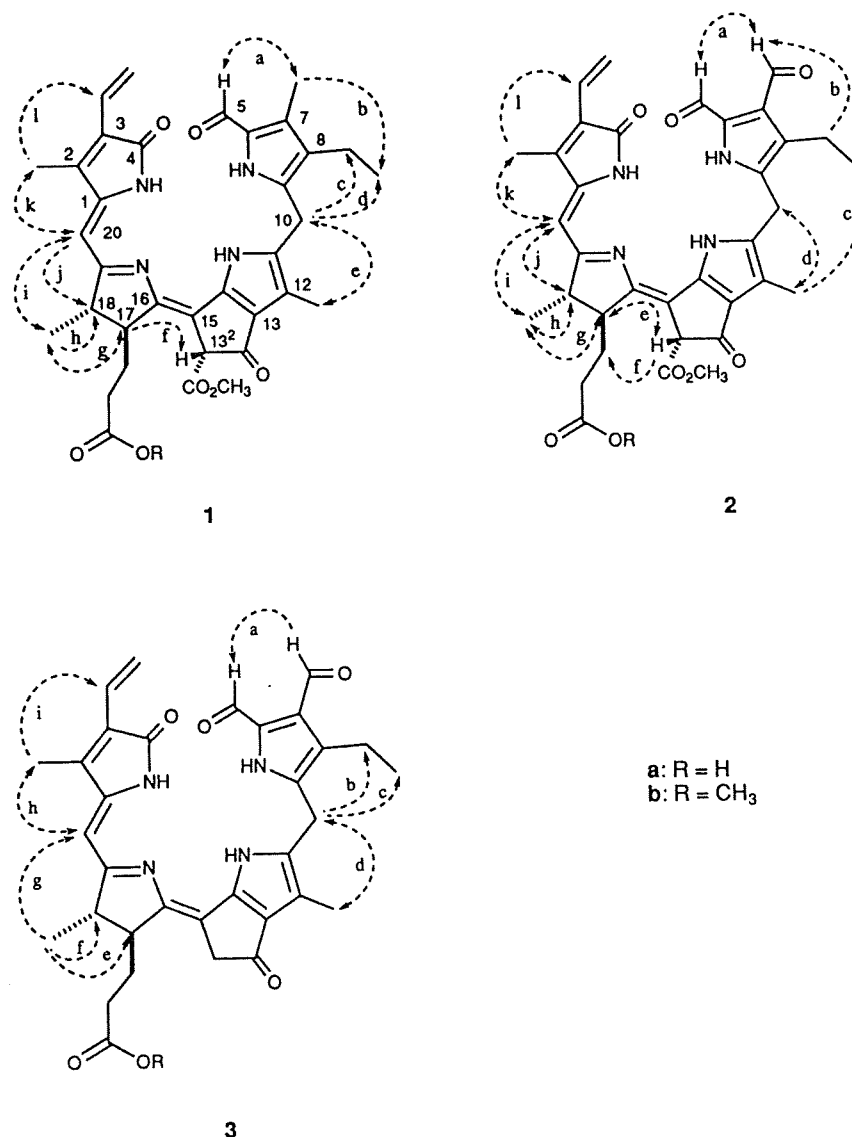
The isolation of a bilin derived from pheophorbide *a* (**1a**) from the culture medium of *C. protothecoides* proves that oxidative cleavage of the tetrapyrrole macrocycle takes place previous to decarbomethoxylation at C-13². Most likely, the latter reaction occurs outside the cells, as a consequence of the slight acidic conditions (pH 6.8) of the culture medium. Another important conclusion of the present work is that enzymatic degradation of both

*Part 5 in the series 'Chlorophyll Catabolism'. For Part 4 see ref. [1].

†Dedicated to Professor Wolfhart Rüdiger on the occasion of his 60th birthday.

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§As usual when handling chlorophyll derivatives, some epimerization on C-13² takes place during the process of isolation of **1b** and **2b** as it is revealed by the ¹H NMR spectra of both compounds (cf. [7]).



chlorophyll *a* and *b* in bleached *Chlorella* cells takes place by the same mechanism.

EXPERIMENTAL

General methods. All chemicals were reagent grade; solvents were distilled prior to use. TLC plates precoated with silica gel 60 and silica gel 60 PF₂₅₄₊₃₆₆ for the prep. TLC (1.25 mm thick, 20 × 20 cm) were purchased from E. Merck (Germany). The cell density was determined using a Thoma counter chamber with a Zeiss Axioskop microscope. UV and visible spectra were recorded using CH₂Cl₂ solns containing 1% MeOH; λ_{\max} are quoted in nm and relative band intensities in parenthesis. ¹H NMR spectra were measured in CDCl₃. Chemical shifts (δ) are given in ppm downfield from tetramethylsilane, as int. standard, and coupling constants (*J*) in Hz. Resonance signals were assigned either by homonuclear NOE difference experiments (Tables 1–3) or according to their mul-

tiplicities and chemical shifts. High resolution mass spectra (HRMS) were obtained with a Vacuum Generator Micromass 70 70E instrument equipped with a DS 11-250 data system using the fast atom bombardment (FAB) ionization technique with Xe at 8 kV in glycerol-1-thioglycerol solns.

Algal culture. The *C. protothecoides* strain used in this work is the same as previously described [3]. The culture medium specified therein has been slightly modified by addition of 1 ml l⁻¹ of an aq. CaCl₂ soln (150 mg 100 ml⁻¹) to the basal medium (BM). The greening cell cultures were shaken at 25°, under illumination with GRO-LUX (Sylvania GTE, Germany) fluorescent lamps (2000 lux in total), for 7 days. Under these conditions the final cell density amounts to about 10⁸ cells ml⁻¹ and the generation period to 8 hr. The procedure of bleaching of the cells is the same as previously described [3].

Isolation and characterization of the pigments. After sepn of the main pigment [3] and its *E*-isomer [5] from

Table 1. ^1H NMR signals of **1b** assigned by ^1H $\{^1\text{H}\}$ NOE difference experiments

Irradiated frequency	Enhanced signal	% Enhancement	Assignment in formula 1b
9.43	2.22 (3H, s, H-7 ¹)	6.5	a
5.68	2.72 (1H, <i>qd</i> , $J = 7.3$, $J = 1.9$ Hz, H-18)	2.8	j
	2.15 (3H, s, H-2 ¹)	8.6	k
	1.19 (3H, <i>d</i> , $J = 7.3$ Hz, H-18 ¹)	3.4	i
3.95	2.38 (2H, <i>q</i> , $J = 7.6$ Hz, H-8 ¹)	1.5 ^a	c
	2.28 (3H, s, H-12 ¹)	4.7 ^a	e
	0.96 (3H, <i>t</i> , $J = 7.6$ Hz, H-8 ²)	small ^a	d
2.51	4.43 (1H, s, H-13 ²)	1.5	f
	1.19 (3H, <i>d</i> , $J = 7.3$ Hz, H-18 ¹)	4.5	g
2.28	3.97, 3.92 (2H, $2 \times d$, $J = 16.1$ Hz, H-10)	0.9	e
	0.96 (3H, <i>t</i> , $J = 7.6$ Hz, H-8 ²)	0.8	not shown
2.22	9.43 (1H, s, H-5)	2.7 ^a	a
	0.96 (3H, <i>t</i> , $J = 7.6$ Hz, H-8 ²)	1.2 ^a	b
2.15	6.56 (1H, <i>dd</i> , $J = 17.2$, $J = 11.1$ Hz, H-3 ¹)	3.1	l
	5.68 (1H, s, H-20)	3.1	k
1.19	5.68 (1H, s, H-20)	1.8	i
	2.72 (1H, <i>qd</i> , $J = 7.3$, $J = 1.9$ Hz, H-18)	7.9	h
	2.51 (1H, <i>m</i> , H-17)	1.5	g

^aThe neighbouring signals are affected by off-resonance irradiation. The reported values are referred to 100% intensity of the sum of the saturated signals.

Table 2. ^1H NMR signals of **2b** assigned by ^1H $\{^1\text{H}\}$ NOE difference experiments

Irradiated frequency	Enhanced signal	% Enhancement	Assignment in formula 2b
10.27	9.86 (1H, s, H-5)	6.9	a
9.86	10.27 (1H, s, H-7 ¹)	5.7	a
5.71	2.71 (1H, <i>m</i> , H-18)	3.7	j
	2.14 (3H, s, H-2 ¹)	6.3	k
	1.20 (3H, <i>d</i> , $J = 7.3$ Hz, H-18 ¹)	2.2	i
4.44	2.51 (1H, <i>m</i> , H-17)	2.1	e
	1.94 (1H, <i>m</i> , H _B -17 ¹)	2.3	f
4.04	2.31 (3H, s, H-12 ¹)	3.5 ^a	d
2.71	10.27 (1H, s, H-7 ¹)	1.7 ^a	b
	5.71 (1H, s, H-20)	2.5 ^a	j
	2.31 (3H, s, H-12 ¹)	1.0 ^a	not shown
	1.20 (3H, <i>d</i> , $J = 7.3$ Hz, H-18 ¹)	4.3 ^a	h
	1.07 (3H, <i>t</i> , $J = 7.6$ Hz, H-8 ²)	6.5 ^a	not shown
2.51	4.44 (1H, s, H-13 ²)	1.5	e
	1.20 (3H, <i>d</i> , $J = 7.3$ Hz, H-18 ¹)	2.5	g
2.31	4.04, 3.97 (2H, $2 \times d$, $J = 16.1$ Hz, H-10)	small	d
	1.07 (3H, <i>t</i> , $J = 7.6$ Hz, H-8 ²)	0.8	c
2.14	6.52 (1H, <i>dd</i> , $J = 17.9$, $J = 11.0$ Hz, H-3 ¹)	2.2	l
	5.71 (1H, s, H-20)	2.5	k
1.20	5.71 (1H, s, H-20)	1.5	i
	2.71 (1H, <i>m</i> , H-18)	7.9	h
	2.51 (1H, <i>m</i> , H-17)	2.0	g

^aSee footnote in Table 1.

Table 3. ^1H NMR signals of **3b** assigned by ^1H $\{^1\text{H}\}$ NOE difference experiments

Irradiated frequency	Enhanced signal	% Enhancement	Assignment in formula 3b
10.28	9.86 (1H, s, H-5)	4.6	a
5.72	2.16 (3H, s, H-2 ¹)	5.2	h
4.03	2.71 (2H, q, $J = 7.5$ Hz, H-8 ¹)	1.7	b
	2.30 (3H, s, H-12 ¹)	1.7	d
	1.06 (3H, t, $J = 7.5$ Hz, H-8 ²)	1.2	c
2.62	1.70 (1H, m, H _A -17 ¹)	4.2	not shown
2.30	4.03, 3.97 (2H, 2 \times d, $J = 16.2$ Hz, H-10)	1.7	d
2.16	6.52 (1H, dd, $J = 17.3$, $J = 11.3$ Hz, H-3 ¹)	2.9	i
	5.72 (1H, s, H-20)	2.8	h
1.21	5.72 (1H, s, H-20)	1.6	g
	2.72 (1H, m, H-18)	6.9	f
	2.62 (1H, m, H-17)	3.2	e

the culture medium, the red residue was rechromatographed on TLC plates (*vide infra*) using CH_2Cl_2 -MeOH-Me₂CO (16:3:1) as eluant. The top of the main red band still contains a small quantity of the previously characterized bilin [3] and was, therefore, discarded. As a further resolution of the remaining mixt. of pigments was not feasible, the latter was dissolved in tetrahydrofuran and treated successively with a large excess of carbonyl bisimidazole and MeOH containing 1% methanolic potassium methoxide (cf. [5]). Thereafter, the soln was poured into H₂O and extracted with CH_2Cl_2 . The organic layer was sepd, the solvent evapd *in vacuo*, and the residue purified by prep. TLC on silica gel, using CH_2Cl_2 -MeOH-Me₂CO (90:9:1) as eluant, to yield 3 new well-sepd zones. The main component (R_f 0.56) was characterized as the methyl ester **1b** of a bilin derived from pheophorbide *a*. The other pigments (R_f 0.62 and 0.44) were characterized as products of cleavage of pheophorbide *b* (**2b**) and pyropheophorbide *b* (**3b**), respectively.

4,5-Dioxo-4,5-secopheophorbide a methyl ester (1b). UV/vis: 278 (0.65), 312 (1.00), 428 (0.21), 464 (0.27), 496 (0.33), 534 (0.29), 584 (0.14). ^1H NMR: δ 10.22, 10.13 and 8.92 (each 1H, *br s*, NH); 6.36 (1H, *d*, $J = 17.2$ Hz, H_{trans}-3²); 5.55 (1H, *d*, $J = 11.5$ Hz, H_{cis}-3²); 3.76 and 3.64 (each 1H, *s*, OMe); 2.25 (2H, *m*, H-17²); 1.94 and 1.72 (each 1H, 2 \times *m*, H-17¹); further signals in Table 1. HRMS (FAB) m/z 641.2975 [MH]⁺. Calcd for C₃₆H₄₁N₄O₇: 641.2975.

4,5-Dioxo-4,5-secopheophorbide b methyl ester (2b). UV/vis: 274 (0.89), 332 (1.00), 464 (0.34), 494 (0.42), 530 (0.39), 582 (0.18). ^1H NMR: δ 11.15, 10.29 and 8.96 (each 1H, *br s*, NH); 6.34 (1H, *d*, $J = 17.9$ Hz, H_{trans}-3²); 5.55 (1H, *d*, $J = 11.0$ Hz, H_{cis}-3²); 3.77 and 3.64 (each 1H, *s*, OMe); 2.71 (2H, *q*, $J = 7.6$ Hz, H-8¹); 2.25 (2H, *m*, H-17²); 1.71 (1H, *m*, H_A-17¹); further signals in Table 2. HRMS (FAB)

m/z 655.2778 [MH]⁺. Calcd for C₃₆H₃₉N₄O₈: 655.2768.

4,5-Dioxo-4,5-secopyropheophorbide b methyl ester (3b). UV/vis: 278 (0.85), 326 (1.00), 460 (0.36), 492 (0.44), 528 (0.41), 574 (0.23). ^1H NMR: δ 11.05, 10.37 and 8.79 (each 1H, *br s*, NH); 10.28 (1H, *s*, H-7¹); 6.40 (1H, *d*, $J = 17.3$ Hz, H_{trans}-3²); 5.58 (1H, *d*, $J = 11.3$ Hz, H_{cis}-3²); 3.65 (1H, *s*, OMe); 3.51 and 3.30 (each 1H, *d*, $J = 20.1$ Hz, H-13²); 2.27 (2H, *m*, H-17²); 1.97 (1H, *m*, H_B-17¹); 1.21 (3H, *d*, $J = 7.3$ Hz, H-18¹); further signals in Table 3. HRMS (FAB) m/z 597.2718 [MH]⁺. Calcd for C₃₄H₃₇N₄O₆: 597.2713.

Acknowledgements— ^1H NMR and mass spectra were measured by F. Fehr and F. Nydegger, respectively. Financial support of this work by the Swiss National Science Foundation (Project No. 20-30236.90) is gratefully acknowledged.

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152. X-Ray Molecular Structure of a Red Bilin Derivative from *Chlorella protothecoides*

4th Communication on Chlorophyll Catabolism¹⁾

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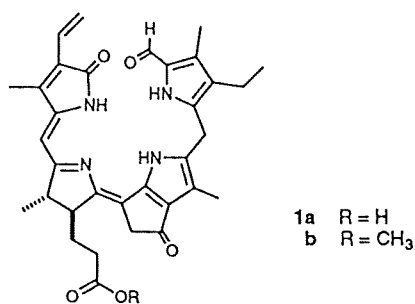
(29.IV.93)

The molecular structure of a chlorophyll *a* catabolite isolated from the culture medium of bleached *Chlorella protothecoides* cells has been determined by X-ray diffraction; the crystal structure shows a distorted helical arrangement of the pyrrole rings and consists of symmetrical dimers located at a crystallographic diad, which are stabilized by six intermolecular H-bonds.

1. Introduction. – In the investigation of the enzymatic degradation of chlorophylls – like in many other domains of molecular biology – the study of a particular microorganism, in this case the microalgae *Chlorella protothecoides*, facilitates substantially the understanding of the same process in more complicated pluricellular organisms. Thus, the elucidation of the structure of the pigments excreted in the culture medium during the process of bleaching of *C. protothecoides* cells, when this green algae is grown in a medium rich in glucose but poor in nitrogen [2], has revealed them as products of degradation of chlorophyll *a* [3] [4] similar to the so-called *RP-14* pigment which has been isolated from dark-bleached excised primary leaves of barley (*Hordeum vulgare* cv. GERBEL) [5].

The present communication deals with an X-ray diffraction study of the molecular structure of the main product of chlorophyll *a* catabolism isolated from *C. protothecoides* which confirms the structure suggested earlier on the basis of analytical data [3].

2. Results and Discussion. – The pigment isolated from the culture medium **1a** was transformed into the corresponding methyl ester **1b** through successive reaction with



¹⁾ Part 3: [1].

Although pigment **1b** is optically active, the absolute configuration at the chiral centers of the molecule could not be determined from the diffraction data; thus the absolute configuration at the chiral centers of the molecule is assumed to be the same as in chlorophyll *a* [7–10].

The present X-ray molecular structure determination is the first one of a natural product of chlorophyll *a* catabolism and confirms the structure already suggested by spectroscopic methods [3] and corroborated by partial synthesis from methyl pyropheophorbide *a* [4].

This work has been supported by the *Swiss National Science Foundation* (project No. 20-30236.90) and by the *Austrian Science Foundation* (project No. 8371).

Experimental Part

X-Ray Diffraction. Crystal data: orthorhombic space group $P2_12_12$, with cell dimensions: $a = 18.995(2)$ Å, $b = 23.831(3)$ Å, $c = 7.403(1)$ Å; $V = 3351.1(11)$ Å³, $Z = 4$, $\rho_c = 1.155$ g/cm³, $F(000) = 1240$. Intensity data were collected using monochromatic $\text{CuK}\alpha$ radiation ($\lambda = 1.5418$) at 88(2) K on a *Siemens P4* diffractometer for 3384 reflections ($2^\circ \leq 2\theta \leq 113.5^\circ$), of which 3168 were unique ($R_{\text{int}} = 2.14\%$). A semi-empirical absorption and volume (Ψ -scan) correction was performed. The structure was solved by direct methods, 1718 reflections with $F > 4\sigma(F)$ were used for refinement. Anisotropic atomic displacement parameters (a.d.p.'s) were refined for the four N-atoms and most of the peripheral atoms; scattering factors were taken from the *International Tables for X-Ray Crystallography* [11]. The R index after full-matrix refinement [12] was 10.2% ($R_w = 8.36\%$, $1/\sigma^2$ weights) for a data-to-parameter ratio of 6.0:1. H-Atoms attached to N(1), N(2), and N(3) were observed from difference electron density *Fourier* syntheses; the same is true for some of the Me protons. The positions of the remaining H-atoms were calculated on the basis of stereochemical plausibility. During the refinement, H-atoms were treated as 'riding' on the respective non-H-atom, with fixed isotropic a.d.p.'s. Refinement was terminated when Δ/σ for all parameters refined was considerably below 1, with the exception of the parameters of the pseudo atoms included to account for the disordered solvent density. These atoms showed Δ/σ values up to 3.0.

Atomic coordinates, bond lengths and angles, and thermal parameters have been deposited at the *Cambridge Crystallographic Data Centre*.

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CHLOROPHYLL CATABOLISM. PART 3. STRUCTURE ELUCIDATION AND PARTIAL SYNTHESIS OF A NEW RED BILIN DERIVATIVE FROM *Chlorella protothecoides**

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Abstract—The structure of a new red pigment produced during the “bleaching” of *Chlorella protothecoides* cells has been elucidated by analytical methods as well as by *in vitro* isomerization into the new compound of a synthetic sample of a previously characterized chlorophyll *a* catabolite.

INTRODUCTION

When grown in a medium rich in glucose or acetate but poor in nitrogen, the microalgae *Chlorella protothecoides* excretes several red pigments into the culture medium that were thought some years ago to be products of degradation of chlorophylls.¹ Recently, the peculiar structure **1a** of the main component in this mixture of red pigments has been elucidated by analytical methods¹ and confirmed by a six-step partial synthesis starting from chlorophyll *a*.²

The present work deals with the isolation and characterization of a further red pigment (**2a**), which is present in much lower quantities (about 10% of pigment **1a**) in the culture medium of “bleached” *C. protothecoides* cells. In some cultures the amount of the new pigment in proportion to **1a** was increased, thus suggesting that the latter is transformed into **2a** either enzymatically within the cells or chemically in the culture medium. As a matter of fact, **2a** proved to be less prone to decomposition than **1a** under the conditions used for their isolation and purification. An important contribution to the elucidation of the origin of the new pigment was made by the study of the chemical properties of **1a** and its methyl ester **1b**, as both became readily accessible by chemical synthesis.²

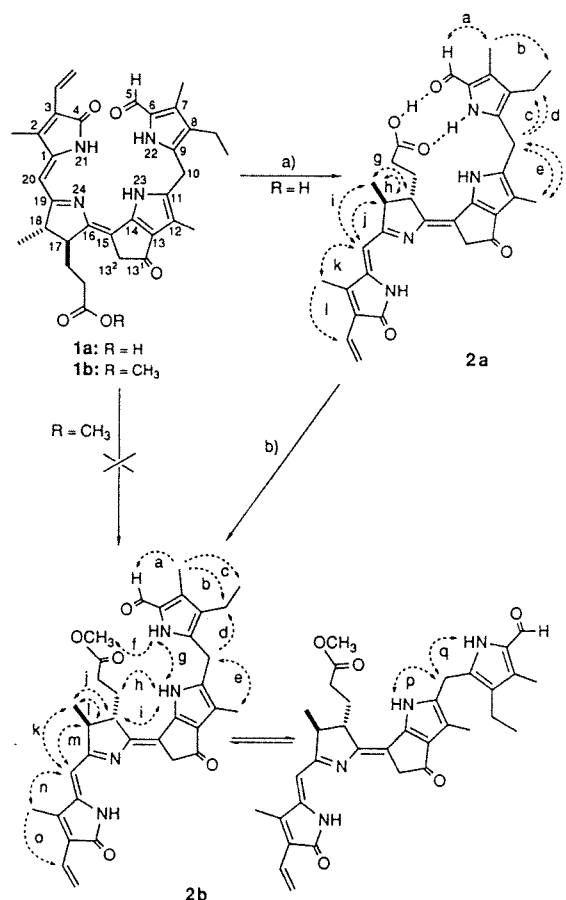
MATERIALS AND METHODS

The *C. protothecoides* strain used in this work is the same as previously described for the isolation of pigment **1a**.¹ The culture medium has been slightly modified for the scaling-up of the process. The cells were cultivated in blade-stirred Bioengineering AG bioreactors. The greening process was performed in a glass fermentor equipped with a light jacket, the bleaching phase in a stainless steel fermentor. The use of such 10 L bioreactors at PRTM, F. Hoffmann-La Roche, AG, CH-4002 Basel, enabled the isolation of the new pigment from 8 L cultures in substantially higher quantities than from the laboratory-scale experiments described previously.¹

All chemicals were reagent grade; solvents were distilled prior to use. Thin-layer chromatography (TLC) plates precoated with silica gel 60 and silica gel 60 PF₂₅₄₋₁₀₀ for the preparative TLC (1.25 mm

thick, 20 × 20 cm) were purchased from E. Merck (D-6100 Darmstadt).

Ultraviolet and visible spectra were recorded with a Perkin-Elmer 320 spectrophotometer using CH₂Cl₂ solutions containing 1% methanol; λ_{max} are quoted in nm and band intensities in parentheses, as log ϵ . Emission spectra were measured with a Perkin-Elmer MPF-4 fluorescence spectrophotometer. ¹H NMR spectra were performed by F. Fehr at 360.13 MHz with a Bruker AM-360 instrument equipped with an Aspect 3000 data system. Chemical shifts (δ) are given in ppm downfield from tetramethylsilane, as internal standard, and



a) : CH₂Cl₂ - piperidine, 20°C, 20 min, 25%.
 b) : (i). 1,1'-carbonyldiimidazole in THF, 5 h. (ii). Methanol - 1% methanolic sodium methoxide, 2 h, 92 %

*Parts 1 and 2 have been published in *FEBS Letters*¹ and *Tetrahedron*,² respectively.

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‡Abbreviations: FAB, fast atom bombardment; MS, mass spectra; NOE, nuclear Overhauser effect; TLC, thin-layer chromatography.

Table 1. ^1H NMR signals of compound 2a assigned by $^1\text{H}(^1\text{H})$ -NOE difference experiments*

Irradiated frequency	Enhanced signal†	% Enhancement	Label in formula 2a
9.33	2.27 (s, $\text{CH}_3(7')$)	4.5	a
5.76	2.79 (m, $\text{CH}(18)$)	3.5	j
	2.16 (s, $\text{CH}_3(2')$)	6.8	k
	1.22 (d, $J = 7.3$, $\text{CH}_3(18')$)	1.9	i
3.99	2.48 (q, $J = 7.5$, $\text{CH}_2(8')$)	2.5	c
	2.28 (s, $\text{CH}_3(12')$)	5.0	e
3.90	2.48	4.9	d
	2.28	5.2	f
2.79	5.76 (s, $\text{CH}(20)$)	2.8‡	j
	2.64 (m, $\text{CH}_B(17^2)$)	2.9‡	Not shown
	1.28 (m, $\text{CH}_A(17')$)	3.1‡	Not shown
	1.22	3.8‡	g and h
2.28	3.99 (d, $J = 15.6$, $\text{CH}_B(10)$)	1.3‡	e
	3.90 (d, $J = 15.6$, $\text{CH}_A(10)$)	1.2‡	f
2.27	9.33 (s, CHO)	2.8‡	a
	1.04 (t, $J = 7.5$, $\text{CH}_3(8^2)$)	1.2‡	b
2.16	6.57 (dd, $J = 17.7$, $J = 11.5$, $\text{CH}(3')$)	3.7‡	l
	6.30 (dd, $J = 17.7$, $J = 1.8$, $\text{CH}_{trans}(3^2)$)	1.4‡	Not shown
	5.76	3.2‡	k
	1.28	2.8‡	Not shown
1.22	5.76	1.8‡	i
	2.79 (m, $\text{CH}(17)$ and $\text{CH}(18)$)	6.0‡	g and h
	2.18 (m, $\text{CH}_B(17')$)	2.2‡	Not shown

* 3.3×10^{-3} M in CDCl_3 - CD_3OD (2:1).

†The other resonance signals were assigned according to their multiplicities and chemical shifts: 5.54 (dd, $J = 11.5$, $J = 1.8$, $\text{CH}_{cis}(3^2)$); 3.70 and 3.61 ($2 \times \text{d}$, $J = 20.6$, $\text{CH}_2(13^2)$); 2.50 (m, $\text{CH}_A(17^2)$).

‡The neighboring signals are affected by off resonance irradiation. The reported values are referred to 100% intensity of the sum of the saturated signals.

coupling constants (J) in Hertz. Spin multiplicities are indicated by symbols s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). High-resolution mass spectra (MS) were obtained by F. Nydegger with a Vacuum Generator Micromass 70 70E instrument equipped with a DS 11-250 data system using the fast atom bombardment (FAB) ionization technique with Xe at 8 kV in glycerol/1-thioglycerol solutions.

(15E)-4,5-dioxo-10,22-dihydro-4,5-secopyropheophorbide (2a). A solution of 4 mg of synthetic 1a² in 0.4 mL of CH_2Cl_2 -piperidine (2:1) was kept at room temperature for 20 min in the dark and then poured into water (200 mL). The mixture was acidified with 32% aqueous HCl (3 mL) and extracted with CH_2Cl_2 . The organic layer was separated, the solvent evaporated *in vacuo* and the residue purified by preparative TLC using CH_2Cl_2 -acetone-methanol (80:16:4) as eluant to yield 1.8 mg (45%) of starting material (R_F 0.3) and 1 mg (25%) of 2a (R_F 0.55). λ_{max} : 314 (4.45), 426 (3.89), 458 (4.08), 486 (4.23), 528 (4.24), 578 (3.98). ^1H NMR: see Table 1.

(15E)-4,5-dioxo-10,22-dihydro-4,5-secopyropheophorbide methyl ester (2b). Into a solution of 2a (2 mg) in tetrahydrofuran (3 mL) a large excess (30 mg) of 1,1'-carbonyldiimidazole (Fluka Chemie AG, CH-9470 Buchs) was added at once, and the mixture was allowed to stand at room temperature for 5 h before 1 mL of methanol and 4 drops of 1% methanolic sodium methoxide were added. After a further 2 h the solution was poured into water (200 mL) and extracted with CH_2Cl_2 . The organic layer was separated, the solvent evaporated *in vacuo* and the residue purified by preparative TLC using CH_2Cl_2 -acetone-methanol (80:18:2) as eluant to yield 1.8 mg (92%) of 2b. λ_{max} : 310 (4.45), 424 (3.92), 454 (4.12), 486 (4.26), 526 (4.27), 5.74 (4.00). ^1H NMR: see Table 2.

RESULTS AND DISCUSSION

The red pigment 2a isolated from *C. protothecoides* was transformed into the corresponding methyl ester by the same procedure used for the synthetic material (*cf.* Materials and Methods). The identity of the two preparations was con-

firmed *inter alia* by their high-resolution FAB MS, which established the molecular formula to be $\text{C}_{34}\text{H}_{38}\text{N}_4\text{O}_5$ for both esters (calculated for $[\text{C}_{34}\text{H}_{38}\text{N}_4\text{O}_5]^+$: 583.2915, found: 583.2924 and 583.2928 for the natural and synthetic samples, respectively). Moreover, the ^1H NMR spectrum of a mixture of the natural and synthetic pigments proved to be identical with the ^1H NMR spectra of the individual components. Evidence for structure 2b, which is assigned to the methyl ester of the new red pigment isolated from *C. protothecoides* cultures was obtained from a series of nuclear Overhauser effect (NOE) difference experiments depicted in formula 2b (*cf.* Table 2). Thus, contrary to ester 1b in which NOE correlations between $\text{CH}_{\text{proR}}(13^2)$ and $\text{CH}(17)$ as well as $\text{CH}_{\text{proS}}(13^2)$ and one of the protons on C(17') had been found previously,² the intensity enhancement of the signals assigned to the H atom on C(17) as well as to the $\text{CH}_2(17')$ and the OCH_3 groups of the propionic acid chain, observed in the spectrum of 2b on irradiation at the resonance frequency of the pyrrolic NH protons, are consistent with the E geometry of the $\text{C}(15) = \text{C}(16)$ bond of the latter. On the other hand, the finding that irradiation at the resonance frequency of the pyrrolic NH protons enhances also the intensity of the singlet assigned to the methylene H atoms on C(10), as well as the fact that these H atoms are isochronous, manifest the free rotation on the $\text{C}(9) - \text{C}(10)$ and $\text{C}(10) - \text{C}(11)$ bonds in 2b. Owing to the insolubility of the carboxylic acid 2a in aprotic solvents, corresponding NOE correlations could not be established for this compound in solvents in which exchange of the protons of the HN groups by $^2\text{H}^+$ takes place. Therefore, the assignment of the structure of 2a is based, in

Table 2. ^1H NMR signals of compound **2b** assigned by $^1\text{H}(^1\text{H})$ -NOE difference experiments*

Irradiated frequency	Enhanced signal†	% Enhancement	Label in formula 2b
10.80	9.69 (broad s, NH(22))	5.2	g
	3.93 (s, CH ₂ (10))	1.6	p
	2.68 (m, CH(17))	9.8	i
	2.18 (m, CH _β (17'))	5.0	h
9.69	10.80 (broad s, NH(23))	3.7	g
	4.01 (s, OCH ₃)	3.6	f
	3.93	0.9	q
5.60	2.72 (m, CH(18))	3.8	m
	2.11 (s, CH ₃ (2'))	8.3	n
	1.20 (d, $J = 9.7$, CH ₃ (18'))	2.4	k
3.93	10.80	1.7	p
	9.69	2.1	q
	2.49 (q, $J = 7.5$, CH ₂ (8'))	2.8	d
	2.28 (s, CH ₃ (12'))	3.9	e
2.70	5.60 (s, CH(20))	3.1‡	m
	1.33 (m, CH ₄ (17'))	2.4‡	Not shown
	1.20	6.5‡	j and l
2.26	9.49 (s, CHO)	3.3	a
	2.49	0.6	b
	1.07 (t, $J = 7.5$, CH ₃ (8'))	0.6	c
2.11	6.55 (dd, $J = 17.6$, $J = 11.3$, CH(3'))	3.5	o
	6.39 (dd, $J = 17.6$, $J = 2.0$, CH _{trans} (3'))	0.5	Not shown
	5.60	3.9	n
1.20	5.60	2.0	k
	2.68 (m, CH(17)) and 2.72 (m, CH(18))	9.6	j and l

* 5.0×10^{-3} M in CDCl_3 .†The other resonance signals were assigned according to their multiplicities and chemical shifts: 9.89 (broad s, NH(21)); 5.54 (dd, $J = 11.3$, $J = 2.0$, CH_{cis}(3')); 3.70 and 3.62 ($2 \times$ d, $J = 20.6$ CH₂(13')); 2.72–2.55 (m, CH₂(17')); 2.26 (s, CH₃(7')).

‡The neighboring signals are affected by off resonance irradiation. The reported values are referred to 100% intensity of the sum of the saturated signals.

the first instance, on the assumption that no unexpected molecular changes occur on esterification of the latter. Actually, this assumption is corroborated by the high similarity of both the absorption and emission spectra of **2a** and its methyl ester **2b** (*cf.* Fig. 1). Moreover, when measured in CDCl_3 containing CD_3OD the methylene H atoms on C(10) of **2a** give rise to an AB spin system at δ 3.99 and 3.90 ppm ($J = 15.6$ Hz), as would be expected when free rotation of the formylpyrrole moiety is hindered to some extent by the intramolecular hydrogen bonding depicted in formula **2a** (*cf.* Table 1). As mentioned before, the CH₂(10) group of **2b** is observed both in CDCl_3 and in CDCl_3 containing CD_3OD as a singlet (at δ 3.93 and δ 3.96 ppm, respectively).

The conversion of carboxylic acid **1a** in its isomer **2a** has been observed in different solvents like CH_2Cl_2 , methanol, acetone, tetrahydrofuran and acetonitrile. In all of them the conversion rate hardly reached 3–5% after 48 h at room temperature in the dark. The yield of the reaction could be slightly increased in a shorter time by irradiation with daylight, but degradation of the pigment **1a** becomes more important under these conditions. In acetic acid or dimethylformamide as solvents, **1a** is transformed largely into a yellow pigment, the structure of which is, at present, under investigation. Until now, the highest yield of the transformation of **1a** in **2a** has been obtained in CH_2Cl_2 solutions containing piperidine at room temperature in the dark. Under these conditions up to 25% of **2a** was obtained after 20 min and 45% of starting material was retrieved. Increasing the reac-

tion time to 60 min the yield of **2a** could be improved up to 30% but the amount of recovered **1a** decreased to 15% while a number of additional fluorescent red pigments were formed as high-polar by-products. Surprisingly, in the presence of bases other than piperidine (pyridine, triethylamine, diethyl-

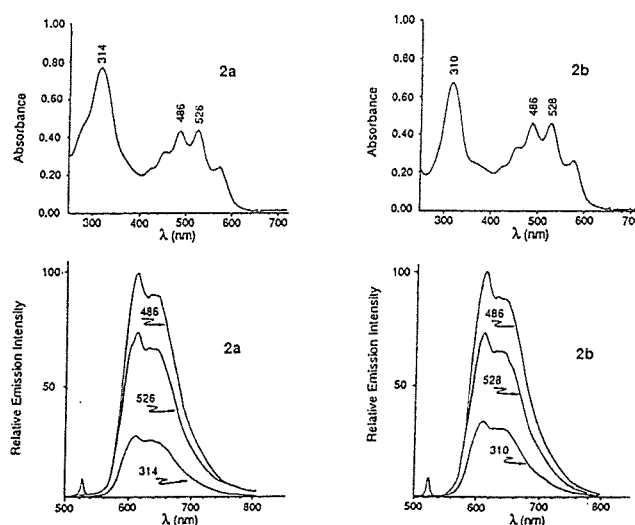


Figure 1. Absorption and fluorescence (at 610 nm) spectra of pigments **2a** and **2b** both 2.5×10^{-3} M in CH_2Cl_2 containing 1% methanol. Excitation wavelengths are indicated at the curves.

amine, 1,8-diazabicyclo[5.4.0]undec-7-ene) a number of unidentified by-products were formed. On the other hand, methyl ester **1b** is not transformed appreciably into the corresponding E isomer **2b** under the reaction conditions examined for the isomerization of **1a**. Thus, in piperidine solution the formation of only a very small amount of **2b** could be detected. Under the basic conditions of hydrolysis of **1b** to **1a**,² less than 5% of **2a** is obtained, as a by-product.

The mechanism of the isomerization of **1a** into **2a** is not straightforward. As the transformation takes place in the dark, a photoisomerization of the C(15) = C(16) bond must be ruled out. Taking into account that both the lactam ring (cf. Falk *et al.*⁴) and the hydrogenated C(17)=C(18) bond increases bond fixation in the tripyrrone chromophore of **1a** (cf. Cullen *et al.*⁵), the rotation barrier for the C(15)=C(16) bond should be too high for a thermal isomerization at an appreciable rate. Possibly, however, either the sterically crowded neighborhood of the C(15) = C(16) bond or its situation, which becomes conjugated to the double bond of the enolized CO(13') group, facilitates the Z/E isomerization of the former. On the other hand, as the ratio **2a/1a** in the culture medium of *C. protothecoides* is generally higher than in the *in vitro* experiments, which were carried out with synthetic **1a** dissolved in the same culture medium used for cell growth, an enzymatic transformation of **1a** into **2a** (e.g. through reduction and subsequent dehydrogenation of the

C(15) = C(16) bond) cannot be ruled out from the experimental facts so far available.

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Chlorophyll catabolism in *Chlorella protothecoides*

Isolation and structure elucidation of a red bilin derivative

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When grown in a medium rich in glucose but poor in nitrogen, the algae *Chlorella protothecoides* excrete a red pigment the structure of which has been proven to correspond to a product of oxydative ring cleavage of the chlorophyll-*a* chromophore at the C4–C5 methine bridge.

Chlorophyll-*a* catabolism; 19-Formyl-1[21H, 22H]bilinon derivative

1. INTRODUCTION

Despite of its enormous significance both in plant physiology and environmental sciences, the fate of chlorophylls in senescent chloroplasts is still an enigma [1–4]. The major contribution on this research area has been done just recently by Matile et al. [5–9], who have shown that the first steps of chlorophyll breakdown in senescent barley leaves parallel, to some extent, the degradation of haeme in vertebrates. Our interest in chlorophyll catabolism in *Chlorella* emerged from the observation made some time ago by Oshio and Hase, that these green algae, when grown in a medium rich in glucose or acetate but poor in nitrogen (urea), excrete certain red pigments into the incubation medium, the formation of which proceeds simultaneously with the disappearance of chlorophyll from the algal cells [10]. As, to the best of our knowledge, the structures of these red pigments produced during the 'bleaching' of *Chlorella* cells have not been further investigated previously, Hase's work has been reproduced in our laboratory resulting in the isolation of a bilin derivative structurally related to chlorophyll *a*.

2. EXPERIMENTAL

The studies reported in the present work were carried out with material isolated from cultures of the algae *Chlorella protothecoides*, which were a gift from Prof. Horst Senger (Universität Marburg, Germany). The same strain had been used earlier by E. Hase et al. [11], and was originally supplied from the Algal Culture Collection at the University of Indiana (USA) labeled as ACC no. 25. The culture medium employed was essentially the same as described in [10] but modified in order to maintain a constant pH of 6.8 during incubation

and to control the level of available nitrogen. Thus, $(\text{NH}_4)_2\text{HPO}_4$ was used, instead of urea, as nitrogen source, since we found that in sterile filtered preculture media containing urea, rather limited growth of the above strain was detectable. On heat sterilization of the urea containing medium, however, free ammonia, which is traceable with Nessler's reagent, is generated by hydrolysis, thus explaining the normal growth of the cells reported in [10] under these conditions.

The basal medium (BM) used contained per l: K_2HPO_4 (8.7 g), KH_2PO_4 (6.8 g), $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ (0.3 g), FeSO_4 (0.01 M, 1 ml, acidified with 5 ml of 2 N H_2SO_4) and 1 ml of oligoelement solution ARNON A5 [12]. Preculture solutions (100 ml) were prepared by heat-sterilizing the BM together with $(\text{NH}_4)_2\text{HPO}_4$ (1.0 M, 0.2 ml). After addition of 10% (w/v) aqueous glucose (10 ml), which had been previously sterilized by heating, and of 0.1 ml of Nitsch and Nitsch vitamin solution (Sigma), the preculture medium was inoculated with *C. protothecoides* cells, taken from a standard agar slant, and shaken in the dark at 25°C for 7–15 days. Greening cell cultures were prepared by adding 5 ml of the above preculture to 100 ml of BM containing 1.5 ml of $(\text{NH}_4)_2\text{HPO}_4$ (1.0 M) and 0.1 ml of vitamin solution. The cultures were shaken at 25°C under illumination with white fluorescent light (4000 lx) for 5–10 days. Thereafter, the cells were centrifuged and washed twice with 50 ml of 0.1 M potassium phosphate buffer (pH 6.8). Bleaching cell cultures were obtained when the washed cells were transferred to 100 ml BM, containing 0.1 ml of vitamin solution and 10 ml of 10% aqueous glucose, and the cultures were shaken at 25°C in the dark. After 50 h the red nutrient broth was separated from the cells by centrifugation and filtration of the supernatant over a thin layer of Cellite. Algae cells can be recycled for the greening process after washing with sterile buffer solution. To isolate the red pigments from the culture medium, an aliquot (1 l) of the above filtrate was shaken with 100 ml of diethyl ether, the organic layer was discarded, and the aqueous phase was extracted repeatedly with diethyl ether (3 × 200 ml), after addition of 10 g of citric acid. The combined organic phases were repeatedly washed with 1% aqueous citric acid (5 × 100 ml), dried shortly over Na_2SO_4 and the solvent was removed on a rotary evaporator. After separation of the mixture of pigments by preparative thin layer chromatography (PTLC) on silica gel 60 (Merck) using $\text{CHCl}_3/\text{CH}_3\text{OH}$ (95:5) as eluant, the main product (R_F 0.35) was extracted from the stationary phase with ethanol and further purified by repeated precipitation from a conc. CHCl_3 solution with a large excess of *n*-hexane. The precipitate was separated by filtration through a cotton plug, washed with *n*-hexane, and dried in vacuo to yield ca. 1 mg of the pure pigment (1a) with UV/vis [λ_{max} (rel. intensity) in CH_3OH]: 320 (1), 455 (0.25), 490 (0.38), 525 (0.36), and 580 (0.2).

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The purified pigment was transformed into the corresponding methyl ester as follows: to a solution of **1a** in dry tetrahydrofuran (2 ml) carbonyldiimidazole (50 mg) was added. The mixture was allowed to stand at 25°C for 24 h and, thereon, 2 ml of CH₃OH, containing 3 drops of a 1% solution of CH₃ONa in CH₃OH, were added. After 5 h, the reaction mixture was diluted with 30 ml of diethyl ether and shaken with 50 ml of 5% aqueous citric acid. The organic phase was washed with water, dried over Na₂SO₄, and the residue obtained after evaporation of the solvent under reduced pressure was purified by PTLC as above. The product (*R_F* 0.7) was extracted from silica gel with ethanol and dried in vacuo to yield **1b** with UV/vis [λ_{\max} (rel. intensity) in CH₃OH]: 315 (1), 450 (0.33), 483 (0.39), 525 (0.33) and 580 (0.19).

3. RESULTS

The structures of the red pigment from *C. protothecoides* and its methyl ester (**1a** and **1b**, respectively) were elucidated by spectroscopic analysis. A first hint was given by the pattern of the ¹H-NMR spectrum of **1a** in CD₃OD as solvent, which shows all characteristic signals for the peripheric substituents of pyropheophorbide-*a*. However, all signals are high field shifted with respect to those of the latter, thus suggesting that π -electron delocalization in the macrocycle has been interrupted either by reduction of bridging double bond(s) or by cleavage of the macrocycle at one of the methine bridges. Accordingly, a Soret band at λ_{\max} ca. 400 nm is absent in the UV/vis spectrum of **1a**. Therefore, the down field singlet at δ 9.36 ppm has to be assigned to an aldehydic H-atom rather than to a methine proton. Although in CDCl₃ as solvent the ¹H-NMR spectrum is significantly less resolved, three additional broad singlets corresponding to NH groups appear at δ 9.7, 10.0, and 10.5 ppm. Moreover, the diastereotopic H atoms at C10, which in CD₃OD give rise to a singlet at δ 4.05 ppm, are manifested by an AB-system with *J* = 15.6 Hz in CDCl₃. The presence of a carboxylic acid group in the molecule of **1a** is evidenced by the fact that the pigment can be dissolved in aqueous Na₂CO₃ and extracted, after acidification with citric acid, with an organic sol-

vent. As expected, after esterification a singlet at δ 3.56 ppm is present in the ¹H-NMR spectrum of **1b**. The bulk structure of **1a** was convincingly confirmed by the high resolution FAB mass spectrum of the corresponding methyl ester (**1b**), which established the molecular formula to be C₃₄H₃₈N₄O₅ (calc. for [C₃₄H₃₉N₄O₅]⁺: 583.2915; found: *m/z* 583.2926 (MH)⁺; Xe at 8 kV in a glycerol/1-thioglycerol matrix). Thus, compared with methyl pyropheophorbide-*a* (C₃₄H₃₆N₄O₃), the molecular formula of **1b** contains two atoms of oxygen and two atoms of hydrogen more. The number of H-atoms corresponds to that found in the ¹H-NMR spectrum. Evidence concerning the site at which the putative biogenetic precursor of **1a**, pyropheophorbide-*a*, has been oxidatively cleaved was furnished by the characteristic pattern of the ABX system assigned to a vinyl group adjacent to a lactam carbonyl in the bile pigment series [13]. Moreover, the same pattern has been observed in the 9,10-dehydro derivative of **1b**, which has been characterized recently (as the Cd complex) in our laboratory as

Table I

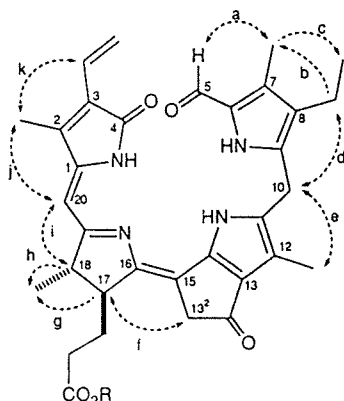
¹H-NMR signals of compound **1a** assigned by ¹H{¹H}-NOE difference experiments

Irradiated signal ^a	Enhanced signal ^b	% Enhancement	Assignment in formula 1a ^c
9.36	2.25 (s, 3 H-7 ¹)	3.7	a
6.63	2.19 (s, 3 H-2 ¹)	2.7	k
6.03	2.19	5.3	j
	2.86 (qd, <i>J</i> = 7.3 & 1 Hz, H-18)	3.3	i
4.05	2.03 (s, 3 H-12 ¹)	2.3	e
	2.42 (q, <i>J</i> = 7.6 Hz, 2 H-8 ¹)	1.3	d
3.35	2.63 (dm, <i>J</i> = 9 Hz, H-17)	small	f
2.86	1.19 (d, <i>J</i> = 7.3 Hz, 3 H-18 ¹)	2.5	h
	6.03 (s, H-20)	3.3	i
2.63	1.19	2.5	g
	3.35 (d, <i>J</i> = 20.4 Hz, H _{pro-R} -13 ²)	small	f
2.42	2.25	1.0	b
	4.05 (s, 2 H-10)	0.9	d
2.25	0.95 (t, <i>J</i> = 7.6 Hz, 3 H-8 ²)	2.5	c
	9.36 (s, H-5)	2.6	a
2.19	6.03	1.4	j
	6.63 (dd, <i>J</i> = 17.6 & 11.5 Hz, H-3 ¹)	2.7	k
2.03	4.05	0.6	e

^a δ in ppm (CD₃OD, 360.13 MHz) referred to CHD₂OD (δ = 3.30) as internal standard.

^b The other resonance signals were assigned on the base of their multiplicity and chemical shifts: 6.32 (dd, *J* = 17.6 & 2.1 Hz, H_{trans}-3²), 5.51 (dd, *J* = 11.5 & 2.1 Hz, H_{cis}-3²), 3.60 (d, *J* = 20.4 Hz, H_{pro-S}-13²), 2.23 (m, 2 H-17²), 1.93 and 1.64 (2m, 2 H-17¹).

^c Corresponding signals for **1b**: 9.48 (s, H-5), 6.61 (dd, *J* = 17.7 & 11.6 Hz, H-3¹), 6.35 (dd, *J* = 17.7 & 2.4 Hz, H_{trans}-3²), 6.05 (s, H-20), 5.53 (dd, *J* = 11.6 & 2.4 Hz, H_{cis}-3²), 4.05 (s, 2 H-10), 3.65 (s, OCH₃), 3.55 (d, *J* = 20.5 Hz, H_{pro-S}-13²), 3.35 (d, *J* = 20.5 Hz, H_{pro-R}-13²), 2.83 (qd, *J* = 7.2 & 1.5 Hz, H-18), 2.63 (ddd, *J* = 9.4, 3.4 & 1.5 Hz, H-17), 2.43 (q, *J* = 7.6 Hz, 2 H-8¹), 2.37 (dd, *J* = 7.8 & 7.0 Hz, 2 H-17²), 2.26 (s, 3 H-7¹), 2.21 (s, 3 H-2¹), 2.04 (s, 3 H-12¹), 1.98 (ddt, *J* = 14.0, 7.8 & 3.4 Hz) and 1.68 (ddt, *J* = 14.0, 9.4 & 7.0 Hz) (2 H-17¹), 1.19 (d, *J* = 7.2 Hz, 3 H-18¹), 0.97 (t, *J* = 7.6 Hz, 3 H-8²).



1a R = H
1b R = CH₃
 Fig. 1.

the main product of the in vitro photooxidation of the cadmium chelate of methyl pyropheophorbide-*a* [14]. At last, structure **1a** has been confirmed by a series of NOE correlation experiments which are depicted on formula **1** (cf. Table I). Despite substantial efforts on NOE measurements, however, an interaction between H-13² and H-17 could not be conclusively established (cf. ref. [15]). Nevertheless, considering both the probable biogenesis of **1a** and the unequivocal correspondence of the chemical shifts assigned to the ¹H-atoms in the proximity of the C15-methine bridge of **1b** with those of the Cd complex of its 9,10-dehydro derivative [14], the geometry of the bridging olefinic bond between C15 and C16 is assumed to be *Z*. On the other hand, the same absolute configurations found in chlorophylls [16,17] are tentatively assigned to the asymmetric atoms C17 and C18.

4. DISCUSSION

Structure **1a** of the red biline derivative excreted into the incubation medium by 'bleaching' *Chlorella protothecoides* cells resembles that of a chlorophyll catabolite recently isolated from chloroplasts of senescent barley leaves [18]. This similarity is particularly interesting as both on the basis of theoretical considerations [19] and of the structure of some luciferins isolated from bioluminescent plancton [15,20] electrophilic attack at the methine bridges adjacent to the reduced pyrrole ring had been predicted as the initial step of enzymatic chlorophyll breakdown (cf. ref. [21]). Though almost 30 years ago, in the porphyrin series, it was claimed that a formylbilinone analogous to **1a** had been obtained by cleavage of the haeme chromophore by a liver enzyme [22,23], the structure of the isolated product could not be confirmed later [24]. Thus, compound **1a** together with the chlorophyll-*a* catabolite reported in ref. [18] represents the first examples of natural products, whose structures correspond to those of the photooxidation products of porphyrins and chlorins in vitro [24].

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to Hase's work as well as for having provided us with cells of *Chlorella protothecoides*. We thank Prof. Ph. Matile (Institut für Allgemeine Botanik der Universität Zürich, Switzerland) for communication of his results prior to publication.

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